Biomarkers predictive of clinical outcome in chronic myeloid leukaemia

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by

Claire Marie Lucas

November 2011

Abstract

Imatinib has undoubtedly revolutionised the treatment of chronic myeloid leukaemia (CML) and hence has become first-line treatment. At the time of carrying out this work, the only data available on the efficacy of imatinib for newly diagnosed CML patients were from a single clinical trial, IRIS. In a complete population study in our geographical area, following two years of imatinib treatment, the complete cytogenetic response (CCR) rate was only 51%, which is considerably worse than IRIS.

Clinical decisions would be greatly facilitated if it was possible to accurately predict a patient's probability of achieving a CCR, and in particular to identify those destined to subsequently progress to blast crisis while on imatinib treatment. In identifying patients likely to achieve a CCR on imatinib treatment the most promising biomarker investigated was the pCrkL/CrkL ratio. 100% of patients with a pCrkL/CrkL ratio of less than 25 achieved a CCR, whilst no patient with a ratio greater than 25 achieved a CCR; the latter all required a change in treatment. A patient's *BCR-ABL1* transcript type can provide additional information, but neither this or the pCrkL/CrkL ratio are predictive of blast crisis.

The role of *ALOX5* was investigated for the first time in human CML. In contrast to published data in a mouse model, *ALOX5* expression appears down-regulated in CML due to low levels of the LTB4 receptor BLT1. These data suggest caution when extrapolating mouse model data to human CML.

PP2A is a phosphatase and tumour suppressor which regulates cell proliferation, differentiation and survival. The predictive value of assessing PP2A and/or its inhibitory proteins was investigated. CIP2A is a novel inhibitor of PP2A. Patients with a high CIP2A protein level at diagnosis have a 100% probability of progressing to blast crisis, with the mean time to progression being 13 months; this contrasts with a zero progression rate in patients with low diagnostic CIP2A protein levels. CIP2A acts by inhibiting PP2A, resulting in the stabilisation of c-Myc. Via its role in cell cycle promotion and cellular proliferation, c-Myc may then contribute to disease progression by promoting aneuploidy. In summary, CIP2A is a prospective biomarker of blast crisis in CML and may be a useful therapeutic target. As a result of this study identification of patients who will subsequently progress into blast crisis is now possible.

Acknowledgements

I would like to thank both my supervisors Professor Richard Clark and Dr Robert Harris. Without their support, belief and guidance this degree would have not been possible, for which I am very grateful.

There are many individuals within the Section of Haematology who deserve my thanks. Some have contributed scientific input and discussion, whilst others have simply lent time and listened. In particular I would like to thank Dr Athina Giannoudis for her help and support.

All of this work would not have been possible without the support and encouragement of my family. My heartfelt thanks go to my husband Chris, my Mum and my sister Stephanie.

Declaration

This thesis is the result of my own work, except were indicated. The TaqMan qRT-PCR assays were performed by Dr Athina Giannoudis. hOCT1 mRNA expression experiments were performed by Dr Athina Giannoudis and Mrs Gemma Austin. The LTB4 ELISA assay was performed in collaboration with Miss Elizabeth McDonald. The BCR-ABL1 protein FACS assay was performed in collaboration with Miss Jemma Fagan.

Table of contents

Publications

1. **Lucas CM**, Harris RJ, Giannoudis A, Copland M, Slupsky JR, Clark RE. Cancerous inhibitor of PP2A (CIP2A) at diagnosis of chronic myeloid leukemia is a critical determinant of disease progression. **Blood**. 2011;117(24):6660-6668.
2. **Lucas CM**, Fagan JL, Carter A, Swale B, Evans C, Clark RE, Harris RJ. Rapid diagnosis of chronic myeloid leukemia by flow cytometric detection of BCR-ABL1 protein. **Haematologica**. 2011;96(7):1077-1078.
3. **Lucas CM**, Harris RJ, Giannoudis A, Davies A, Clark RE. SET Binding Protein 1 expression does not predict clinical outcome in chronic myeloid leukemia. **eBlood**. 2010.
4. **Lucas CM**, Harris RJ, Giannoudis A, Davies A, Knight K, Watmough SJ, Clark RE. Chronic myeloid leukaemia patients with the e13a2 BCR-ABL fusion transcript have inferior responses to imatinib than e14a2 patients. **Haematologica**. 2009;94(10):1362-1367.
5. **Lucas CM**, Harris RJ, Giannoudis A, Knight K, Watmough SJ, Clark RE. BCR-ABL1 tyrosine kinase activity at diagnosis, as determined via the pCrkL/CrkL ratio, is predictive of clinical outcome in chronic myeloid leukaemia. **British Journal of Haematology**. 2010;149(3):458-460.
6. **Lucas CM**, Wang L, Austin GM, Knight K, Watmough SJ, Shwe KH, Dasgupta R, Butt NM, Galvani D, Hoyle CF, Seale JRC, Clark RE. A population study of imatinib in chronic myeloid leukaemia demonstrates lower efficacy than in clinical trials. **Leukemia**. 2008;22(10):1963-1966.

Presentations

European Haematology Association annual conference 2011 – London, UK

1. Cancerous inhibitor of PP2A (CIP2A) at diagnosis of chronic myeloid leukaemia is a critical determinant of disease progression.

   Lucas CM – Oral presentation

2. Cancerous inhibitor of PP2A (CIP2A) inhibits PP2A and stabilises PIM1 and c-Myc in CML leading to blast crisis.

   Lucas CM – Oral presentation

3. *ALOX5* is down-regulated at diagnosis of chronic myeloid leukaemia.

   Lucas CM, Harris RJ, McDonald E, Giannoudis A, Holcroft AK, Fowler RC Clark RE

European school of Haematology annual CML meeting 2010 – Washington, USA

Cancerous Inhibitor of PP2A (CIP2A) at diagnosis of chronic myeloid leukaemia is a critical determinant of disease progression.

Lucas CM – Oral presentation

European Haematology Association annual conference 2010 – Barcelona, Spain

The pre-treatment levels of Protein Phosphatase 2A (PP2A) and its inhibitor SET can predict patients destined to progress to blast crisis of CML.

Lucas CM, Harris RJ, Clark RE.

British Society of Haematology annual conference 2010 – Edinburgh, UK

The clinical significance of Protein Phosphatase 2A (PP2A) in CML.

Lucas CM, Harris RJ, Clark RE.

European school of Haematology annual CML meeting 2009 – Bordeaux, France

Initial diagnostic BCR-ABL tyrosine kinase activity determined via the pCrkL/CrkL ratio is predictive of long term treatment outcome in chronic myeloid leukaemia (CML).

Lucas CM, Harris RJ, Giannoudis A, Knight K, Watmough SJ, Clark RE.

European Haematology Association annual conference 2009 – Berlin, Germany

Chronic myeloid leukaemia patients with the e13a2 BCR-ABL transcript have inferior response to imatinib than e14a2 patients.

Lucas CM, Harris RJ, Wang L, Knight K, Watmough SJ, Clark RE.

British Society of Haematology annual conference 2009 – Brighton, England

FACS assessment of pCrkL/CrkL ratio at initial diagnosis is predictive of long term treatment outcome in chronic myeloid leukaemia (CML).

Lucas CM, Harris RJ, Giannoudis A, Knight K, Watmough SJ, Clark RE.

British Society of Haematology annual conference 2008 – Glasgow, Scotland

A population study of imatinib in chronic myeloid leukaemia (CML) demonstrates lower efficacy than in clinical trials.

Lucas CM, Wang L, Austin GM, Knight K, Watmough SJ, Shwe KH, Dasgupta R, Butt NM, Galvani D, Hoyle CF, Seale JRC, Clark RE.

Abbreviations

Abbreviation	
5-HEPTE	5(S)-hydroperoxy-6-trans-8,11,14-cis-eicosatetraenoic acid
5-LOX	ALOX5 protein
ABCB1 (MDR1)	ATP-Binding Cassette Sub-Family B Member 1
ABCC1	ATP-Binding Cassette Sub-Family C Member 1
ABCG2	ATP-Binding Cassette Sub-Family G Member 2
ABL1	The human homologue of the Abelson murine leukaemia virus
AGS	Human gastric cancer cell line
ALL	Acute Lymphoblastic Leukaemia
ALOX5	Arachidonate 5-lipoxygenase
AML	Acute Myeloid Leukaemia
AP	Accelerated Phase
Ara-C	Cytosine arabinoside
ASH	American Society of Hematology
BAD	BCL-2-Associated Death Promoter
B-ALL	B-cell Acute Lymphoblastic Leukaemia
BC	Blast Crisis
BCR	Breakpoint Cluster Region
BD	Becton Dickinson
BLT1	LTB4 receptor
cAMP	Cyclic Adenosine Mono-Phosphate
CCR	Complete Cytogenetic Response
CCRe	Complete Cytogenetic Response equivalence
CEBPα	CCAAT/enhancer-binding protein-α

CHR	Complete Haematological Response
CIP2A	Cancerous Inhibitor of PP2A
CML	Chronic Myeloid Leukaemia
CMML	Chronic Myelomonocytic Leukaemia
CMR	Complete Molecular Response
CP	Chronic Phase
CrkL	CT10 regulator of kinase like
DASISION	Clinical trial comparing dasatinib and imatinib in newly diagnosed CP patients.
EFS	Event Free Survival
EHA	European Haematology Association
ELN	European Leukaemia Network
ENESTnd	Clinical trial comparing nilotinib and imatinib in newly diagnosed CP patients.
EUTOS	EUropean Treatment Outcome Study
FBC	Full Blood Count
FISH	Fluorescent *In Situ* Hybridisation
FLAP	Five Lipoxygenase Activating Protein
hOCT1	Human Organic Cation Transporter 1
IFN-α	Interferon-α
IKZF1	Ikaros gene
IRIS	International Randomized Study of Interferon-α and STI571
JAK2	Janus Kinase 2
K562	First human immortalised CML cell line
KCL22	CML cell line
KYO1	CML cell line
LAMA84	CML cell line

LSC	Leukaemic Stem Cell
LTA4	Leukotriene A4
LTB4	Leukotriene B4
MAPK	Mitogen Activated Protein Kinase
M-BCR	Major Breakpoint Cluster Region
m-BCR	Minor Breakpoint Cluster Region
MFI	Mean Fluorescence Intensity
MMR	Major Molecular Response
MNC	Mononuclear cells
NICE	National Institute of Clinical Excellence
No-CCRe	No Complete Cytogenetic Response equivalence
NSCLC	Non-Small Cell Lung Cancer
OA	Okadaic Acid
OS	Overall Survival
pCR	Partial Cytogenetic Response
pCrkL	Phosphorylated CT10 regulator of kinase like
PDGFR	Platelet Derived Growth Factor Receptor
PFS	Progression Free Survival
Ph^+	Philadelphia positive
PI3K	Phosphatidylinositol 3-kinases
PIM1	Proviral Integrations of Moloney virus 1
PKC	Protein kinase C
PP1	Protein phosphatase 1
PP2A	Protein Phosphatase 2A
PP2Ac	Protein Phosphatase 2A catalytic subunit

p-Tyr	Phosphorylated tyrosine
qRT-PCR	quantitative Real Time Polymerase Chain Reaction
ROS	Reactive Oxygen Species
SCT	Stem Cell Transplant
SET	PP2A inhibitor
SETBP1	SET binding protein 1
SH1	Src Homology domain 1
SH2	Src Homology domain 2
SH3	Src Homology domain 3
siRNA	Small interfering RNA
STAT5	Signal Transducer and Activator of Transcription 5
TKI	Tyrosine kinase inhibitor
WBC	White Blood Count
WHO	World Health Organisation
Zileuton	5-LOX inhibitor

CHAPTER ONE - General introduction

1.1.0 INTRODUCTION

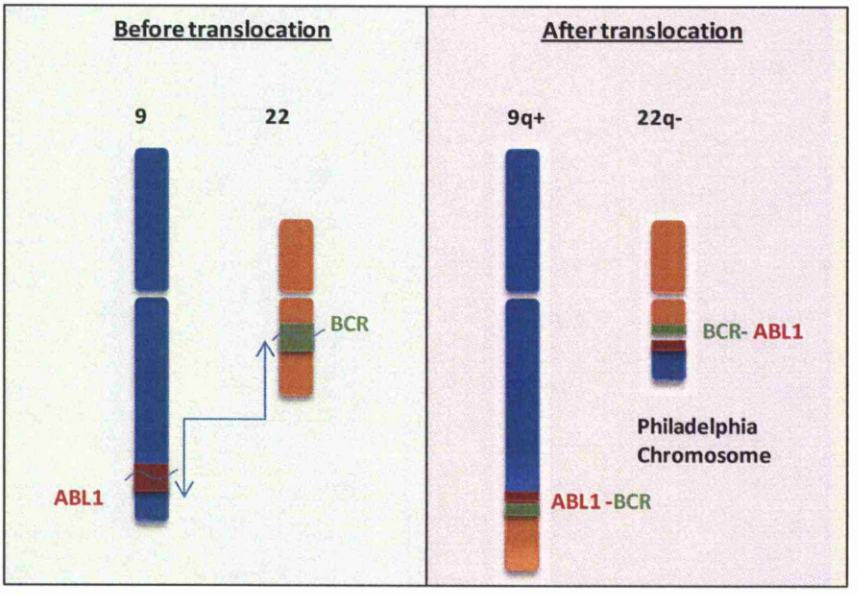
1.1.1. Chronic myeloid leukaemia - a brief history.

In 1845 the first probable case of what we now recognise as chronic myeloid leukaemia (CML) was described by John Hughes Bennett, a doctor from Edinburgh, who initially thought the disease was due to an infection.[1] A few weeks later Virchow in Vienna reported a similar case, hypothesising that it was a non-infectious condition involving white blood cells; hence he named the disease "Weisses Blut" to describe the high proportion of white blood cells observed in the blood.[2] These two reports appeared five weeks apart in the literature of the day, and in 1858 Virchow conceded that Bennett was first to report the disease. It has since been speculated that the case reported by Bennett was indeed chronic myeloid leukaemia, while Virchow's was likely to be chronic lymphocytic leukaemia.[3,4] In 1870 Neumann, working with Virchow, identified that the leukaemic cells originated from the bone marrow,[5] and the advent of panoptic staining methods in the 1880s by Ehrlich assisted in the classification of leukaemia into various types.[6] It was now possible to describe the morphological differences between granulocytes and lymphocytes, leading to the characterisation of chronic myeloid leukaemia (chronic granulocytic leukaemia as it was known at the time) as a distinct disorder. It was not for another 80 years until the next major discovery concerning CML was reported.

In 1960, in Philadelphia USA, Nowell and Hungerford observed that patients with CML had a shortened abnormal chromosome (22q-) which they named the "Philadelphia

chromosome".[7] The development of chromosome banding techniques identified the Philadelphia chromosome as a partial deletion of chromosome 22. The Philadelphia chromosome was later identified by Janet Rowley[8] to arise from a reciprocal translocation between chromosomes 9 and 22 (Figure 1.1.). In the early 1980s the two genes disrupted by the translocation were identified. These were the ABL1 gene from chromosome 9 (the human homologue of the Abelson murine leukaemia virus),[9,10] and its translocation partner the "breakpoint cluster region" (BCR) from chromosome 22.[11]

Figure 1.1. The Philadelphia chromosome
The Philadelphia chromosome results when the distal parts of the long arms of chromosome 9 and 22 break and the resultant fragments switch place. The (9,22) translocation forms a short chromosome 22 which is the Philadelphia chromosome (that contains the abnormal BCR-ABL1 fusion gene), and a abnormally long long arm of chromosome 9.



A major advance in the understanding of CML was the demonstration that the BCR-ABL1 fusion protein encoded by the BCR-ABL1 fusion gene had deregulated tyrosine kinase activity when compared to its normal ABL1 counterpart, and that this was correlated with its ability to transform cells to a malignant phenotype.[12] In the early 1990s it was reported that a CML-like disease could be induced in mice transplanted with BCR-ABL1 positive cells.[13,14] This strongly supported the notion that BCR-ABL1 was the cause of the disease and not just a disease marker. Knowledge of BCR-ABL1 deregulated tyrosine kinase activity as the driving force behind the disorder focused attention on BCR-ABL1, as a logical specific therapeutic target.

In 1996 the first highly specific inhibitor of ABL1 tyrosine kinase activity was described by Druker and colleagues.[15] The drug was initially known as CG57148B or STI571 (Ciba-Geigy/Novartis) but is now commonly known as imatinib (trade name Glivec).[16] Imatinib was first used clinically in June 1998. In the year 2000 a phase III clinical trial, IRIS (International Randomized Study of Interferon versus STI571), opened comparing imatinib to the accepted treatment of the time – interferon alpha plus cytosine.[17,18] Imatinib has undoubtedly revolutionised the treatment of CML and hence has become first-line treatment. However, many patients become resistant to or intolerant of imatinib and it is this which concerns both clinicians and scientists and so the history of CML continues to be written.

1.2.0. CML – IN THE CLINIC

1.2.1. Epidemiology

CML has an annual incidence of 1-2 cases per 100,000 of the population.[19-21] It is the most common myeloproliferative disease - accounting for 15-20% of all leukaemia cases. It is more common in men than women and the median age at diagnosis is 60 years.[22] There is no reported geographical or ethnic background which predisposes patients to CML.[23] Exposure to ionising radiation is the only known risk factor. There was an increased incidence of CML following the atomic bombs at Hiroshima and Nagasaki.[24] Patients exposed to the α-emitter thorotrast, (used as a contrast medium in radiology in the 1930s), as well as patients treated with radiation therapy have all demonstrated an increased risk of developing CML.[25] Furthermore, BCR-ABL1 fusion transcripts can be induced *in-vitro* by high dose ionising radiation.[26] In contrast to acute myeloid leukaemia (AML) there is currently no evidence linking the incidence of CML with exposure to organic solvents such as benzene.[27]

1.2.2. Pathogenesis

CML is a clonal myeloproliferative disorder of haemopoietic stem cells; characterised by the reciprocal translocation between chromosomes 9 and 22 (Philadelphia chromosome) which creates the functional fusion gene BCR-ABL1.[8] BCR-ABL1 is a deregulated tyrosine kinase known to play a crucial role in the pathogenesis of CML.[12] The molecular

characteristics of BCR-ABL1 and its activity will be discussed in more detail in section 1.3.0.

1.2.3. Clinical features

Clinically, patients with CML present with a leukocytosis, a left shift in the differential count i.e. more immature cells, and splenomegaly. CML is not limited to the myeloid compartment since the Philadelphia chromosome can be demonstrated in endothelial cells though not in lymphoid cells. High platelet counts are regularly observed but erythrocytosis is rarely seen. Many patients are asymptomatic at diagnosis, though some may experience vague/mild symptoms such as fatigue, weight loss, anaemia, increased susceptibility to infections or joint/bone pain or symptoms attributable to splenomegaly. Priapism and other thrombotic problems are rare and serious presentations. Patients are often diagnosed by a routine full blood count (FBC) performed for other reasons.[28]

CML has traditionally been described as a tri-phasic disease. The three phases are known as chronic phase (CP), accelerated phase (AP) and blast crisis (BC). During CP there is a significant expansion of the myeloid cell compartment - these cells are able to function and differentiate reasonably normally. Without clinical intervention the disease typically progresses to accelerated phase which is defined as the appearance of one of the following: blasts in the blood or bone marrow >15%, or percentage of blasts plus promyelocytes in the peripheral blood or bone marrow ≥30%, or peripheral blood basophils >20%. (There are no reliable criteria for accelerated phase based on platelet

count as it is virtually impossible to distinguish the effects of treatment from the effects of accelerating disease). The third phase of the disease is blast crisis where immature myeloid blood cells dominate the circulation. Blast crisis is defined as blasts in the blood or bone marrow >30%, or appearance of extramedullary involvement (e.g. chloromas), except for hepatosplenomegaly. However, in the tyrosine kinase inhibitor (TKI) era this may no longer be true, as few patients remain in accelerated phase for any length of time, progressing to blast crisis rapidly thus giving the appearance of a biphasic disorder (CP and BC).

1.2.4. Diagnosis

Diagnosis of CML is generally straightforward. Initial indication of the disease is usually based on the characteristics of a FBC and differential white cell count. Typically the WBC is high with an excess of neutrophils and myelocytes. Absolute basophilia and eosinophilia are also a characteristic finding. Confirmation of the disease occurs by two methods; cytogenetic analysis (G-banding, involving identification of the Philadelphia chromosome) and molecular diagnosis, confirming the presence of BCR-ABL1 transcripts.

Although 95% of CML cases are Philadelphia chromosome (or translocation) positive by G-banding cytogenetic analysis, 5% of patients are negative. This is thought to be because the patient has a cryptic BCR-ABL1 rearrangement that is only detectable at the

sub-microscopic level.[29] Failure to demonstrate the presence of the Philadelphia chromosome is thought to be due to insensitivity of the cytogenetic assay or the quality of the blood /bone marrow specimen. In these cases confirmation of diagnosis can be achieved by performing FISH (fluorescent *in situ* hybridisation, using fluorescent probes for BCR and ABL1) or molecular techniques for BCR-ABL1 transcripts. The advantage of chromosome analysis compared to FISH and molecular techniques is that additional chromosome abnormalities can be identified which may offer additional prognostic information.[30]

The presence of the Philadelphia chromosome and/or BCR-ABL1 transcripts confirms a diagnosis of CML. However, if negative, the patient may have atypical CML (although this cannot be positively distinguished). This is a myeloproliferative disorder which presents like CML but with the absence of the Philadelphia chromosome and BCR-ABL1 transcripts. These patients have a poorer prognosis compared to true CML, and have median survival of 18 months.[31] The differential diagnosis should also include chronic myelomonocytic leukaemia (CMML), polycythaemia vera, essential thrombocytosis, myelofibrosis and a leukaemoid reaction.

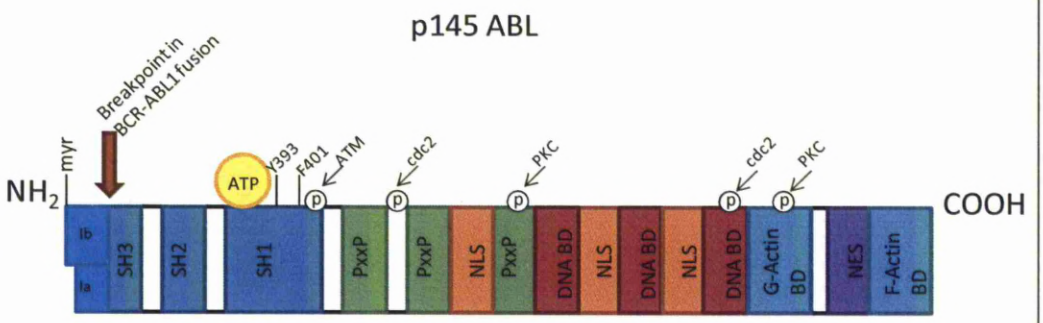
1.3.0. MOLECULAR PATHOGENESIS OF CML

1.3.1. Normal ABL1

The *ABL1* gene is the human homologue of the v-*abl* oncogene carried by the Abelson murine leukaemia virus (A-MuLV).[9] It encodes a non-receptor tyrosine kinase that is expressed in most tissues and is involved in signal transduction, conveying signals from cell surface growth factors and adhesion receptors to regulate cytoskeleton structure, DNA repair and cell cycle regulation.[32,33] Several structural domains have been identified and are shown in Figure 1.2. Three Src homology domains (SH1, SH2 and SH3) are located near the N terminal. SH1 is responsible for tyrosine kinase activity while SH2 and SH3 allow interactions with other proteins. The proline rich region can interact with the SH3 domains of other proteins such as CrkL (CT10 regulator of kinase like).[34]

ABL1 protein can be found in both the cytoplasm and the nucleus of cells and can shuttle between the two compartments under the influence of its nuclear localisation signal (NLS) and its nuclear export signal domains.[35,36] Nuclear ABL1 is an essential pro-apoptotic protein and even though BCR-ABL1 retains the ABL1 nuclear localisation and export signal domains, it is unable to enter the nucleus and therefore exhibits anti-apoptotic properties.[37]

Figure 1.2. Structure of the ABL1 protein
Type Ia isoform is slightly shorter than type Ib, which contains a myristoylation (myr) site for attachment to the plasma membrane. Three Src-homology (SH) domains are located near the NH_2 terminus. Y^{393} is the major site of autophosphorylation within the kinase domain, and phenylalanine 401 (F^{401}) is highly conserved in protein tyrosine kinases which contain a SH3 domain. The proline-rich regions (PxxP) are capable of binding to SH3 domains, and contain the nuclear localisation signals (NLS). The carboxy terminus contains DNA as well as G- and F-actin–binding domains. Sites phosphorylated by ATM, cdc2, and PKC are shown. The red arrow indicates the position of the breakpoint in the BCR-ABL1 fusion protein. (Adapted from Deininger et al[37])



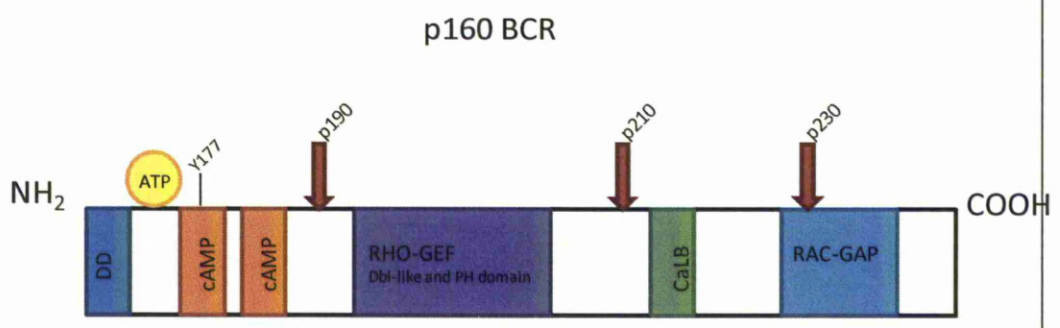
1.3.2. Normal BCR

Normal BCR is expressed in many cell types and the gene is known to be evolutionarily conserved.[38] Its structure is depicted in Figure 1.3. The first N-terminal exon encodes a serine/threonine kinase.[39] The coiled-coil domain at the N terminal allows dimerisation to occur. The BCR protein also contains dbl-like and pleckstrin homology domains (a protein domain of approximately 120 amino acids that occurs in proteins involved in intracellular signalling or as constituents of the cytoskeleton) which stimulate GDP-GTP exchange on Rho guanidine exchange factors,[40] which activate transcription factors such as NF-κB.[41] The C-terminal has GTPase activity for Rac, a member of the RAS family,

which functions to activate NADPH oxidase in neutrophils. Neutrophils from BCR knockout mice have increased reactive oxygen species (ROS) production when activated. Activation also causes an increase in Rac membrane translocation. BCR knockout mice are viable and have a normal phenotype;[42] this may reflect a degree of redundancy with these signalling pathways.[37]

It has been shown in animal models that the BCR component of BCR-ABL1 determines the aggressiveness and lineage of the BCR-ABL1 induced leukaemia.[43] Mutations within the autophosphorylation site Y^{177} of BCR-ABL1 have linked BCR signalling with lineage determination and disease latency.[44] Y^{177} lies within the GRB2 binding motif in BCR and is essential for GRB2/BCR binding.[45] Data suggesting the significance of GRB2/BCR binding are conflicting and controversial. It has been suggested that phosphorylation of BCR-ABL1 at Y^{177} is essential for RAS activation and BCR-ABL1 transformation[45] although others have suggested that RAS is activated by other means.[46]

Figure 1.3. Structure of the BCR protein
At the N-terminal there is a dimerisation domain (DD) and two cyclic adenosine mono-phosphate (cAMP) domains. Y^{177} is the autophosphorylation site crucial for binding to GRB2. The centre of the protein contains a region homologous to Rho guanidine nucleotide exchange factors (Rho-GEF) as well as dbl-like and pleckstrin homology (PH) domains. Toward the C-terminus a putative site for calcium-dependent lipid binding (CaLB) and a domain with activating function for Rac-GTPase (Rac-GAP) are found. Red arrows indicate the position of the breakpoints in the BCR-ABL1 fusion proteins (p190, p210 and p230). (Adapted from Deininger et al[37])



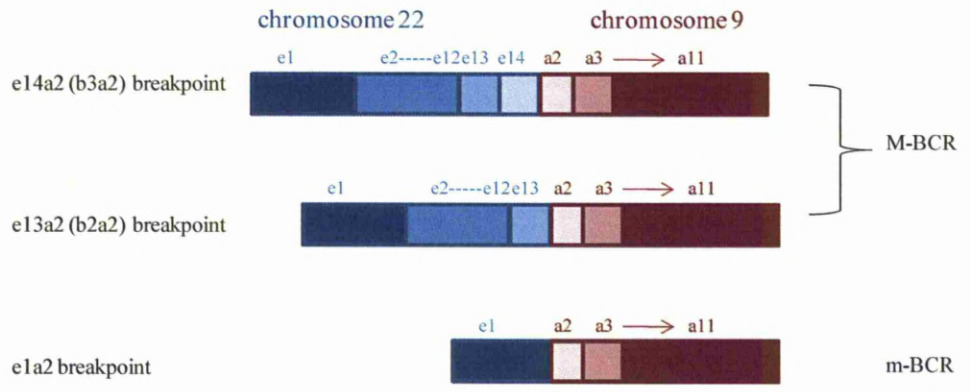
1.3.3. BCR-ABL1

The breakpoints of chromosome 22 cluster within a small (5.8kb) region, spanning exons e12-16 (formerly denoted as b1-5) known as the Major Breakpoint Cluster Region (M-BCR). Breakpoint locations almost always fall between either exons e13 and e14 or between e14 and e15. Although the breakpoints in the ABL1 gene are also variable, because of splicing events, the transcribed mRNA usually has either an e13a2 (b2a2) or an e14a2 (b3a2) junction. The e13a2 and e14a2 *BCR-ABL1* transcript types differ in length by 75bp (25 amino acids).[37] Both *BCR-ABL1* mRNA molecules encode a 210kDa constitutively active protein kinase which is central to the pathogenesis of the disease.[47] Occasionally both major types of BCR-ABL1 transcript (e13a2 and e14a2) can be

detected within the same patient sample and this is thought to be a result of alternative splicing.[48] In addition to BCR-ABL1 being the hallmark of CML it is also found in 25% of patients with B-Cell Acute Lymphoblastic Leukaemia (B-ALL). Two thirds of BCR-ABL1 positive B-ALL patients express an alternative BCR-ABL1 splice variant (e1a2) known as the minor-BCR (m-BCR), which encodes a smaller protein (190kDa). Reports suggest that the p190kDa confers a poorer prognosis than the conventional p210 isoform.[49,50] In rare cases the e1a2 transcript is detected in CML (Figure 1.4).[51]

There is a third breakpoint cluster region known as μ-BCR which is rarely observed in CML.[48] In extremely rare cases breakpoints have been identified outside these three regions.[52,53] Variant BCR-ABL1 transcripts may confer a poor prognosis in imatinib treated patients, although the number of patients reported is small.[52]

Figure 1.4. Breakpoint cluster region of the BCR-ABL1 fusion gene
Breakpoint cluster region of the *BCR-ABL1* fusion gene showing the M-BCR (major) and m-BCR (minor) found in CML.



1.3.4. Does the BCR-ABL1 transcript type offer any additional prognostic information in the imatinib era?

Previous reports prior to the introduction of imatinib have in general not identified an effect of *BCR-ABL1* transcript type on clinical outcome.[54-60] In the imatinib era one small study of 22 patients at different phases of disease suggested that the e13a2 *BCR-ABL1* transcript type may be more sensitive to imatinib treatment;[61] while a larger study has indicated that patients with e14a2 have a better molecular response to imatinib.[62] The clinical significance of the *BCR-ABL1* transcript type in newly diagnosed chronic phase CML patients treated with imatinib remains uncertain. In chapter five I will assess if *BCR-ABL1* transcript type correlates with clinical outcome in a population based study in a single contiguous geographical locality, investigating the clinical outcome of 78 newly diagnosed patients with chronic phase CML treated with imatinib 400mg. In addition, I

will evaluate the significance, if any, of having a variant transcript type in the imatinib era.

1.4.0. BCR-ABL1 TYROSINE KINASE ACTIVITY AND SIGNALLING PATHWAYS

Under normal cellular circumstances the tyrosine kinase activity of ABL1 is tightly regulated. However, when it fuses with BCR to form BCR-ABL1 the BCR part of the protein causes it to be constitutively active.[36] Two adjacent BCR-ABL1 molecules can phosphorylate each other on tyrosine residues in their kinase activation loops.[51] BCR-ABL1 tyrosine kinase activity drives the leukaemic process through the phosphorylation and activation of downstream proteins.[48,50] BCR-ABL1 transforms fibroblasts and haematopoietic cells in culture[63] and induces leukaemia in mice.[13,64,65]

1.4.1. Increased proliferation

Evidence of increased proliferation is derived from the many CML cell lines which were made from primary blast crisis cells and which rapidly proliferate in culture without the need for growth factors. Primary chronic phase cells are able to proliferate rapidly in culture, although they are not completely growth factor independent. When compared to normal cells these cells are able to grow at much lower cytokine concentrations.[66] This may explain why the malignant cells can outgrow normal haematopoietic cells.

BCR-ABL1 can activate the RAS and MAP (Mitogen-Activated Protein) kinase pathways. Phosphorylation of BCR-ABL1 at Y^{177} provides a docking site for the adaptor protein GRB2. GRB2 binds to SOS, which then stabilises RAS in its active GTP-bound form.[45] BCR-ABL1 can also activate the Signal Transducer and Activator of Transcription (STAT) pathway. The STAT family of transcription factors is involved in signal transduction downstream of multiple cytokines, hormones, and growth factors, and is involved in the regulation of cell survival, proliferation, and differentiation.[67] STAT5 has been found to be constitutively active in BCR-ABL1 positive cells, and is associated with anti-apoptotic and proliferative abilities of BCR-ABL1 transformed cells via interaction with the pro-survival protein Bcl-xL.[68] Studies have shown that phosphorylation of Y^{694} within STAT5 is an indicator of BCR-ABL1 activity.[69] However, STAT5 is not immediately downstream of BCR-ABL1, and other signalling pathways converge on STAT5 leading to phosphorylation. STAT5 is therefore not a direct or specific marker of BCR-ABL1 tyrosine kinase activity.

JAK2 functions to transmit signals generated by interaction of IL-3 with the IL-3 receptor. BCR-ABL1 activates JAK2 and inhibition of JAK2 in BCR-ABL1 positive cells can overcome imatinib resistance.[70] Knockdown of JAK2 causes a decrease in the phosphorylation of BCR-$ABL1^{Y177}$ as well as inhibiting the RAS, P13K and STAT pathways.[70]

1.4.2. Reduced apoptosis

BCR-ABL1 stimulates survival pathways resulting in decreased apoptosis (programmed cell death). Several reports have demonstrated that CML cell lines do not undergo apoptosis in response to DNA damage.[71,72] BCR-ABL1 can cause a prolonged rest in the G_2 phase of the cell cycle. This allows cells to extensively undergo DNA repair, resulting in survival of the cells which under normal circumstances would have undergone apoptosis.[71]

Another mechanism by which BCR-ABL1 prevents apoptosis is by preventing the release of cytochrome c from mitochondria and thus the activity of caspases.[73,74] Additionally BCR-ABL1 induces the expression of the anti-apoptotic protein BCL-2, through a signalling mechanism involving RAS.[75] BCR-ABL1 via the AKT/PKB pathways also inhibits apoptosis by phosphorylating the pro-apoptotic protein BAD (BCL-2-associated death promoter). When phosphorylated BAD becomes an anti-apoptotic protein.[76] AKT is a serine/threonine kinase involved in the pro-survival pathway.[77] AKT is recruited to the plasma membrane by binding to phosphatidylinositol 3-kinases (PI3K), and is activated by phosphorylation at Thr308.[69] Activation of AKT is required for BCR-ABL1 transformation of both myeloid and lymphoid lineage cells.[78] AKT activation is mediated in part by the adaptor protein CrkL.[69] CrkL is an adaptor protein, immediately downstream of BCR-ABL1, which mediates BCR-ABL1 signal transduction.[79-81] The role of CrkL as a marker of BCR-ABL1 activity will be discussed in more detail in section 1.6.8.

1.4.3. Genomic instability and DNA repair

Mechanisms responsible for surveying genomic damage and repairing these lesions are compromised in CML, as well as other cancers.[82] BCR-ABL1 induces mutations in genes responsible for maintaining genomic integrity. Chromosome abnormalities such as trisomy 8,[83] trisomy 19,[84] trisomy 21,[83] additional Philadelphia chromosome,[84,85] isochromosome 17,[86] loss of Y chromosome[82,84] and monosomy 7 have all been described in patients progressing to blast crisis;[84] these changes are considered markers of disease progression and an indication of genomic instability. BCR-ABL1 has been shown to induce ROS which results in oxidative DNA damage and double-strand breaks (DSBs) in S and G_2/M cell cycle phases which leads to unfaithful homologous recombination repair (HRR) and nonhomologous end-joining (NHEJ) repair.[87] The ROS generated by BCR-ABL1 can cause kinase domain mutations. Mutations in the BCR-ABL1 kinase domain can cause resistance to imatinib. Inhibition of ROS in leukaemic cells by antioxidants can decrease the frequency of mutations. The combination of imatinib and an antioxidant (pyrrolidine dithiocarbamate (PDTC), N-acetylcysteine (NAC), and vitamin E) reduces the mutagenic frequency in mice.[88] The role of BCR-ABL1 kinase domain mutations will be discussed in more detail in section 1.6.7.

c-Myc is an oncogenic transcription factor which directly or indirectly regulates about 1,000 genes.[89,90] c-Myc regulates cell proliferation, differentiation and stem cell renewal and has been reported to be deregulated in many malignancies including CML.[91-95] Genomic instability is a hallmark of many cancers and c-Myc can cause genomic instability via the induction of ROS and by promoting whole chromosome instability

leading to tetraploidy and aneuploidy.[96] It is known that c-Myc contributes to disease progression in CML, mediated through aneuploidy.[96,97] c-Myc is essential for BCR-ABL1 mediated cellular transformation[98] and has been shown to be over-expressed during blastic transformation.[99] Recently Albajar *et al*[95] demonstrated that c-Myc induces aberrant DNA synthesis in CML cells which are under "imatinib stress." c-Myc mRNA expression levels at diagnosis correlated with treatment response. Furthermore, no correlation between *BCR-ABL1* and *c-Myc* mRNA expression was observed. The role of c-Myc in predicting clinical outcome will be addressed in chapters seven and eight.

The purpose of genomic surveillance is to protect the integrity of the genome. ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia and RAD3-related) nuclear protein kinases function as DNA damage 'sensors'. ATR signalling is inhibited in CML.[100] Following exposure to genotoxic agents or genomic insult BCR-ABL1 translocates to the nucleus, where it binds to both ATM and ATR. ATM is unaffected by BCR-ABL1 binding, as one of its substrates CHK2 (checkpoint kinase 2) can still be phosphorylated.[100] Conversely, ATR is inhibited by BCR-ABL1 binding; evidence for this inhibition is provided by one of its substrates CHK1 (checkpoint kinase 1) which is not phosphorylated and has reduced activity. CHK1 is essential for the activation of the intra-S-phase cell cycle check point.[101] Inhibition of ATR causes inappropriate/uncontrolled DNA replication, despite the presence of DNA damage.[102] Impaired ATR signalling predisposes leukaemic cells to an increased frequency of deletions, mutations and translocations.[103]

1.4.4. Altered adhesion properties

BCR-ABL1 positive progenitor cells exhibit decreased adhesion to bone marrow stroma cells and extracellular matrix.[104] The bone marrow stroma regulates proliferation, and β-integrins are responsible for the interaction between the stroma and progenitor cells. CML cells express a variant of β1 integrin, which has anti-adhesion properties.[105] BCR-ABL1 has also been reported to up-regulate the expression of the α6 integrin.[106] Murine models have demonstrated that mice lacking the adhesion molecules P-selectin and ICAM1 (intercellular adhesion molecule 1) develop a CML-like leukaemia at a significantly faster rate than do wild-type mice. Additionally the authors demonstrated that the lack of P-selectin resulted in early release of BCR-ABL1 expressing myeloid progenitors from bone marrow, appearing to alter the biologic properties of leukaemic cells rather than their growth rate.[107]

1.4.5. Arrest of differentiation

CML progression to blast crisis is characterised by disruption of differentiation. BCR-ABL1 modulates the activity of many transcription factors which regulate genes involved in differentiation. CEBPα (CCAAT/enhancer-binding protein-α) is essential for normal granulocyte differentiation and although present in chronic phase it is lost as patients progress into blast crisis.[108] CEBPα down-regulation is mediated by BCR-ABL1 at the translational level through the stabilisation of the hnRNP E2 (poly(rC)-binding protein heterogeneous nuclear ribonucleoprotein E2). Expression of hnRNP E2 is low in chronic phase but becomes elevated at blast crisis.[108] Ikaros (IKZF1) is essential for early lymphoid lineage commitment.

Deletions in the IKZF1 gene have been associated with disease progression to lymphoid blast crisis.[109] B lymphocytes from mice engineered to express low levels of Ikaros arrest at the pro-B cell stage.[110]

1.5.0. TREATMENT

Several standardised 'milestones' of clinical response have been defined somewhat arbitrarily, to allow comparisons across clinical studies and provide goals for patient responses.[111]

1.5.1. Defining clinical response

Haematological

1. **Complete Haematological Response (CHR)** is defined as the normalisation of the peripheral circulating blood count and resolution of splenomegaly.

Cytogenetic

This is conventionally assessed by G-banded metaphase analysis of at least 20 metaphases from the bone marrow.[111]

- **Minimal Cytogenetic Response (minCR)** = 66-95% Ph^+ metaphases.
- **Minor Cytogenetic Response (mCR)** = 36-65% Ph^+ metaphases.
- **Partial Cytogenetic Response (pCR)** = 1-35% Ph^+ metaphases.
- **Complete Cytogenetic Response (CCR)** = 0% Ph^+ metaphases.

Molecular monitoring is easier and more acceptable to patients than conventional cytogenetics (which requires a bone marrow sample). The correlation between cytogenetically defined CCR and the equivalent *BCR-ABL1/ABL1* transcript ratio has been explored by several groups. In our laboratory, no samples with a *BCR-ABL1/ABL1* ratio of >10% was ever in CCR, but all samples with a ratio ≤1% were in CCR.[112] A ratio of 1% or less can therefore be taken as CCR equivalence (CCRe)[21] and this concept can be useful where only molecular data are available.

Molecular

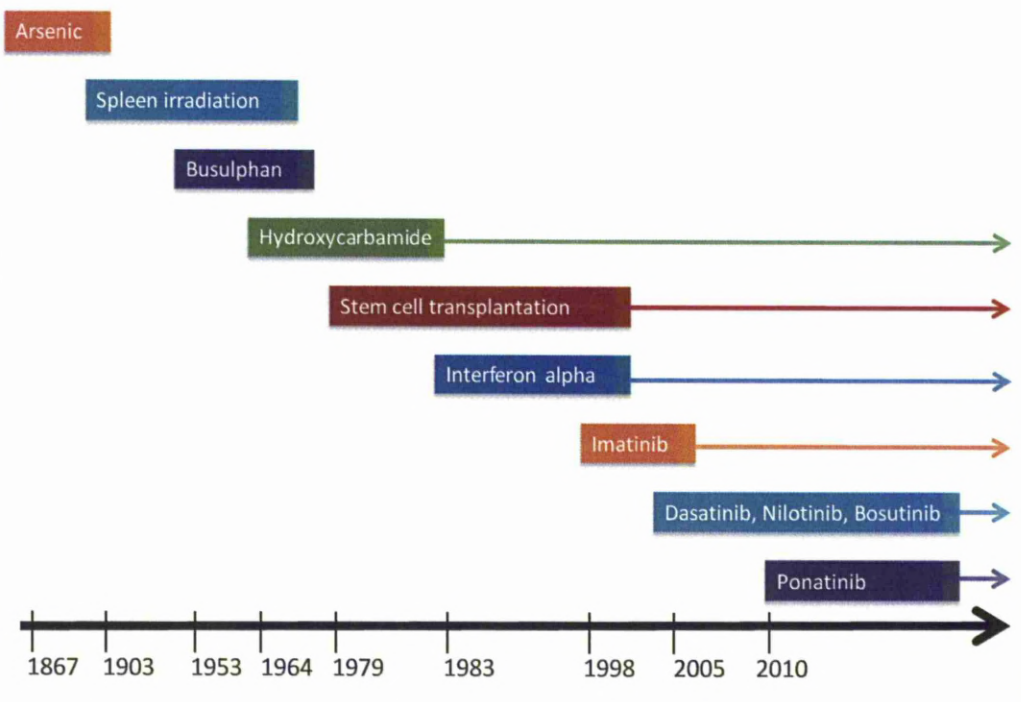
1. **Major Molecular Response (MMR)** is defined as a *BCR-ABL1/ABL* ratio of 0.1% or less. This is sometimes described as a 3 log reduction from baseline/ diagnosis, though this is misleading, since it refers to reduction compared to an arbitrary pool of 30 samples used as a standard for the IRIS (International Randomised Study of Interferon versus STI571) study.[113]
2. **Complete Molecular Response (CMR).** Currently there is no universally agreed definition; however CMR4, CMR4.5 and CMR5 have been suggested referring to 4, 4.5 and 5 log reduction in *BCR-ABL1* transcript ratios respectively.

1.5.2. Treatment in the pre-imatinib era

Arsenic was the first compound used to treat CML in the late 1800s but with the development of radiotherapy arsenic was replaced by splenic irradiation in the 1920s. Although relieving symptoms, this treatment did not increase survival.[23] In the 1950s, the

first synthetic compound used in the treatment of CML was busulphan, an alkylating agent. Busulphan is toxic to stem cells and this may explain the observed increase in survival.[114] Busulphan was replaced in the 1980s by hydroxycarbamide. This was assessed formally as part of a clinical trial which demonstrated a greater overall survival when compared to busulphan.[114] Figure 1.5 shows a time line for the development of treatment for CML.

Figure 1.5. Development of treatment for chronic myeloid leukaemia



Interferon was introduced in 1983, and was standard therapy for CML patients prior to the advent of imatinib. The precise mechanism of action is unknown, however it is thought that interferon may function by selectively targeting the leukaemic cell, enhancing the immune response and regulating haematopoiesis in the bone marrow.[115] Interferon was the first drug to produce cytogenetic responses, varying degrees of which are seen in about one third of patients. Several clinical trials showed that responses to interferon are superior to busulphan or hydroxycarbamide.[114,116,117]

1.5.3. Stem cell transplantation

Stem cell transplantation (SCT) remains the only proven curative therapy for CML.[33,118-120] The guidelines from the American Society of Hematology reported that about 50% of CP CML patients who received a SCT from a matched related donor were alive and leukaemia free at 5 years.[121] Several subsequent reports confirmed these data and extended the follow-up to 10 years, with an overall survival (OS) of 60% and an event-free survival (EFS) of 50%.[33,122,123] Survival after a SCT is dependent on seven risk factors - age, disease phase, transplantation from an unrelated donor, female donor, male recipient, CMV status and time from diagnosis to transplantation.[121,124]

Hehlmann and colleagues[120] compared transplantation to current drug therapy. The study included 621 CP patients. Three hundred and fifty four patients were eligible for transplantation, of which only 123 patients received a transplant due to suitability of

available donors. The 10 year estimated survival for the transplanted group of patients was 53%. Patients who did not have a transplant received interferon treatment until imatinib became available later. The 10 year estimated survival for this group of patients was 52%. Although there was no difference in overall survival at 10 years between the two cohorts, statistical analysis demonstrated that the drug treated group had a superior OS (p=0.049); this is due to the high mortality observed early on in the transplantation cohort. The authors conclude that OS was greater in the drug (interferon/ imatinib) treated cohort and thus drug treatment should be offered as first-line therapy instead of transplantation.[120]

1.5.4. BCR-ABL1 targeted therapy

Since BCR-ABL1 drives the leukaemic process, it is an obvious choice as a therapeutic target.[12,13,23] Initial work focused on inhibition of the tyrosine kinase activity with compounds known as tyrphostins.[125] Tyrphostins compete with ATP and bind to a site outside the active site, affecting BCR-ABL1 activity by a conformational change.[125,126] Although these tyrphostins inhibit growth of the K562 cell line via the inhibition of BCR-ABL1 tyrosine kinase activity, these compounds were not developed for clinical use as these were very toxic.[126]

Another compound which demonstrated activity against BCR-ABL1 is the antibiotic herbimycin A. Herbimycin A acts by accelerating the degradation of the BCR-ABL1

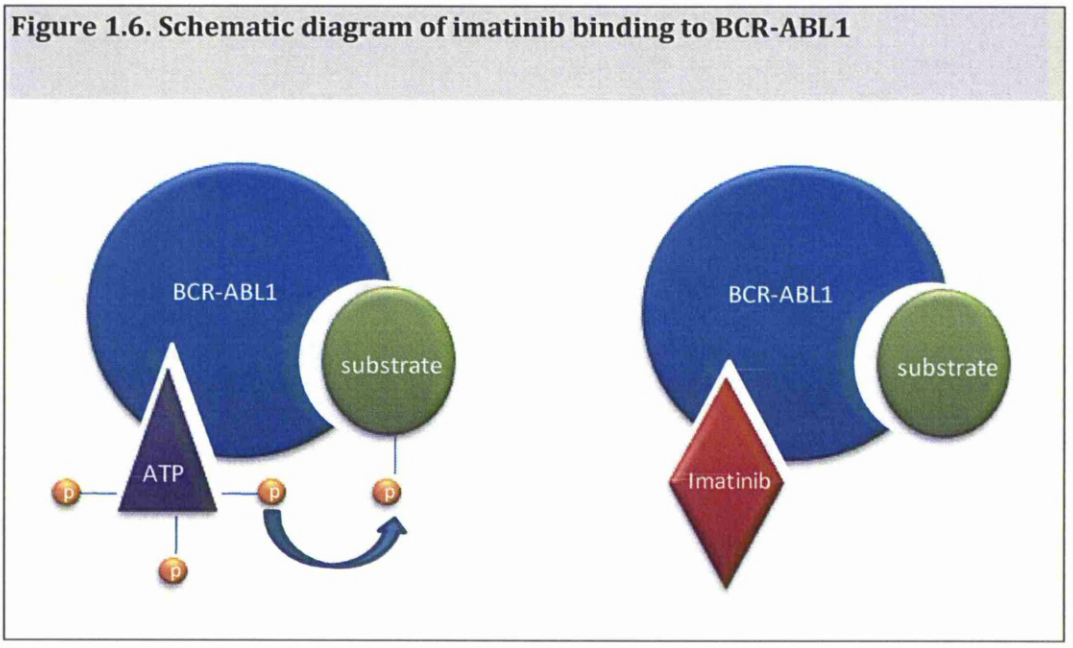
protein but has no effect on the BCR-ABL1 tyrosine kinase activity.[127] Like tyrphostins, herbimycin A was also very toxic and therefore was not developed further for clinical use.

1.5.5. Imatinib

In the late 1980s scientists working for Ciba Geigy, (now part of Novartis) embarked on a project to identify novel PKC (protein kinase C) inhibitors. A 2-phenylaminopyrimidine was identified as the best compound.[128] Although it had low specificity inhibiting both serine/threonine and tyrosine kinases, more specific derivates were synthesised from it. The substitution of a side chain of an aniline phenyl ring with a methyl group enhanced the specificity towards tyrosine kinases and the addition of a benzamide group improved the drug activity towards platelet derived growth factor receptor (PDGFR).[129] Screening of these compounds showed that they also inhibited ABL1.[15,130] The best compound synthesised was CG57148B, later STI571, now known as imatinib, trade name Glivec.[15,128-130]

Imatinib is a tyrosine kinase inhibitor which acts by competing with ATP at the ATP binding site within BCR-ABL1 (Figure 1.6), leading to a decrease in the phosphorylation of proteins downstream of BCR-ABL1 which, in turn, results in several behavioural changes including inhibition of proliferation and enhanced apoptosis as discussed in section 1.4.0.[131]

Figure 1.6. Schematic diagram of imatinib binding to BCR-ABL1

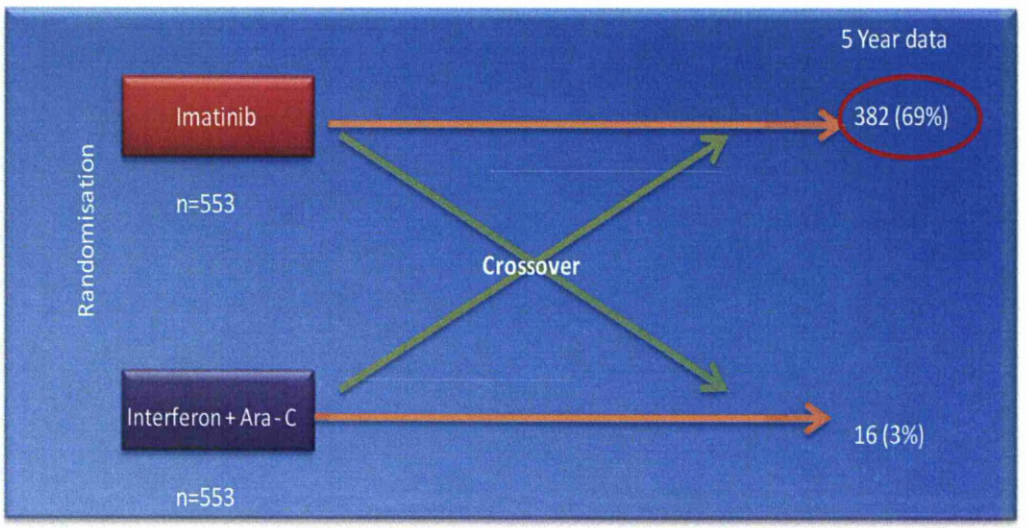


Clinical evaluation of imatinib began in June 1998, when a phase I clinical trial was opened at three centres in the United States. Patients who were resistant or intolerant of interferon were included in this study. The starting dose was 25mg daily; however haematological responses were only seen at doses above 85mg. At a dose of 300mg daily complete haematological responses were seen in 98% of patients, and 31% achieved a major cytogenetic response and 13% achieved a complete cytogenetic response. The observed responses were durable and only 4% of patients lost their response.[132]

Several phase II trials began in 1999 at multiple international centres. These ran in CML patients in myeloid blast crisis, accelerated phase, those who had failed interferon and relapsed Ph+ acute lymphoblastic leukaemia (ALL). The encouraging early data from the

phase I and II trials in chronic phase patients led to the phase III IRIS trial in newly diagnosed chronic phase patients. This opened in the year 2000, comparing imatinib against interferon plus cytosine arabinoside (Ara-C) which at the time was the best available therapy.[133] The trial primary endpoint was time to progression, which was defined as death from any cause during treatment, progression to accelerated phase or blast crisis, extramedullary involvement, loss of CHR or loss of a major cytogenetic response. The secondary endpoints were the rate of complete haematological response, the rate of major and partial cytogenetic response, safety and tolerability.[17] Five hundred and fifty three patients were randomised into each study arm and crossovers were permitted (though tightly policed), if the patient failed to respond to treatment, lost a response, had an increase in the WBC, was intolerant of treatment or had an adverse effect that was considered immediately life-threatening (Figure 1.7).[17]

Figure 1.7. IRIS study design.
Newly diagnosed chronic phase CML patients were assigned to the imatinib or interferon plus Ara–C arm of the trial. Crossover was permitted due to intolerance or failure to respond to treatment. After 5 years of treatment 69% of patients remained on imatinib treatment compared to 3% on interferon + Ara–C.



After five years of treatment 69% of patients assigned to the imatinib arm continued to receive the drug, while only 3% of the patients assigned interferon plus Ara-C remained on treatment. Sixty-five percent of patients originally assigned to receive interferon plus Ara-C switched to imatinib treatment while only 3% of imatinib patients crossed over to receive interferon plus Ara-C.[18] By 18 months of the trial, it was clear that the response rates in the imatinib arm were superior,[17] and it was unethical to continue further follow-up in the interferon plus Ara-C arm. The emphasis of the study therefore changed to become a long-term follow-up study of patients who received imatinib as first-line therapy.

After five years of imatinib treatment the estimated cumulative rate of CHR was 98%. Estimated cumulative CCR rate was 69% at 12 months which increased to 87% at 5 years.[17,18] There was a significant difference in CCR rate according to Sokal score; 89%, 82% and 69% for low, intermediate and high risk groups respectively. The estimated EFS was 83%. Events were defined by the first occurrence of any of the following: death from any cause during treatment, progression to the accelerated phase or blast crisis of CML, or loss of a CHR or MCR.[18] Disease progression to either accelerated phase or blast crisis was estimated to be 6%, which was concentrated in the first 2-3 years of treatment.[18] As a result of the IRIS trial data imatinib 400mg daily became the new standard first-line therapy for newly diagnosed CML patients, approved by the National Institute of Clinical Excellence (NICE) in late 2003.

Imatinib is well tolerated with side effects and toxicity only being reported in a small percentage of patients. Common side effects include oedema, muscle cramps, diarrhoea, nausea, rash and other skin problems, abdominal pain, fatigue, joint pain, and headache. Side effects are generally mild and rarely reach World Health Organisation (WHO) grade 3 or 4 toxicity criteria.[17,18] In the IRIS study haematological toxicity (neutropenia, thrombocytopenia and anaemia) was the most common adverse event.[123] In 2006, *in vitro* imatinib was reported to damage cardiac myocytes and an association was made with clinical cardiac failure.[134] However, a large number of subsequent studies investigating the relationship between imatinib treatment and congestive heart failure have reported no correlation.[135,136]

The IRIS clinical trial results are impressive. However is it important to establish if the IRIS trial results can be reproduced in a more general, unselected, CML population in order to determine the degree of imatinib failure. This will be addressed in chapter three.

1.5.6. Imatinib dose escalation

Dose escalation of imatinib from the standard 400mg daily to 600mg or 800mg daily is a possible treatment option to overcome resistance.[137] The rationale behind this came from cell line work on the overexpression of BCR-ABL1.[137] Treatment with a higher dose of imatinib can induce CHR in 65% of patients who were in haematological relapse and MCR in 56% of patients.[137] The Australian TIDEL (Therapeutic Intensification in DE-novo Leukaemia) trial tested an initial imatinib dose of 600mg daily with a dose escalation to 800mg for sub-optimal responders. For patients receiving 600mg of imatinib the MMR rates were 55% and 77% at 12 and 24 months compared to 32% and 53% for patients receiving less than 600mg.[138]

Recently, the French Spirit trial (STI571 Prospective Randomized Trial) demonstrated that imatinib 600mg is better than 400mg at 12 months, with a CCR rate of 65% versus 58% and an MMR rate of 49% versus 38% for the 600mg and 400mg doses respectively. This trial also showed that the combination of imatinib 400mg and interferon gave even better CCR and MMR rates at 12 months (66% and 57% respectively).[139] However, the German CML IV trial demonstrated a higher rate of MMR at 12 months in patients treated with imatinib 800mg (59% compared to 44% for imatinib 400mg), while showing

no benefit of adding interferon.[140] The British SPIRIT 1 trial also compared these treatments, and has yet to report.

1.5.7. ELN recommendations

In 2006 the European LeukemiaNet (ELN) reviewed all available data in respect to treatment and monitoring of CML patients.[33] The aim was to update definitions of responses and to provide recommendations for the clinical management of CML patients. These recommendations were updated in 2009.[111] These suggest frequent initial monitoring to detect a haematological response e.g. every 15 days. Bone marrow specimens should be sent for cytogenetic analysis at diagnosis, at 3 and 6 months, then every 6 months until a CCR has been achieved, and then every 12 months if robust molecular monitoring is not assured. Whilst these are recommendations and not guidelines, the practicality of performing a bone marrow cytogenetic analysis frequently may be difficult to perform. Cytogenetic analysis certainly should be performed at diagnosis to confirm the presence of the Philadelphia chromosome, as well as to identify additional chromosome abnormalities. It should again be performed at 12 months and when there is an indication of disease progression/ loss of response.[111] Molecular monitoring of *BCR-ABL1* transcripts should be performed every three months as this technique is more accurate and sensitive than cytogenetic analysis in patients with low level disease. Once an MMR has been achieved the ELN recommendations are for monitoring to be performed every 6 months.[111] However, frequent monitoring of patients will allow poor responders/ loss of response to be identified as early as possible.[141]

A summary of ELN criteria for identification of optimal response, sub-optimal response, failure and additional warning signs is shown in Table 1.1.[111]

Table 1.1. Evaluation of response to imatinib treatment.

Time	Optimal	Suboptimal	Failure	Warning signs
Diagnosis	N/A	N/A	N/A	High risk Sokal score. Additional clonal abnormalities in Ph^+ cells
3 Months	CHR and mCR (Ph+ ≤ 65%)	No cytogenetic response (Ph+ >95%)	Less than CHR	N/A
6 Months	pCR (Ph+ ≤ 35%)	Less than PCR (Ph^+ > 35%)	No cytogenetic response (Ph+ >95%)	N/A
12 Months	CCR	pCR (Ph^+ > 35%)	Less than pCR (Ph^+ > 35%)	Less than MMR
18 Months	MMR	Less than MMR	Less than CCR	N/A
At any time	Stable or improving MMR	Loss of MMR Mutation	Loss of CHR. Loss of CCR Mutation Additional clonal abnormalities in Ph^+ cells	Any rise in transcript level Additional clonal abnormalities in Ph^+ cells

Abbreviations
N/A= Not applicable, CHR = Complete Haematological Response, mCR = Minor Cytogenetic Response, pCR =partial Cytogenetic Response, CCR = Complete Cytogenetic Response, MMR = Major Molecular response

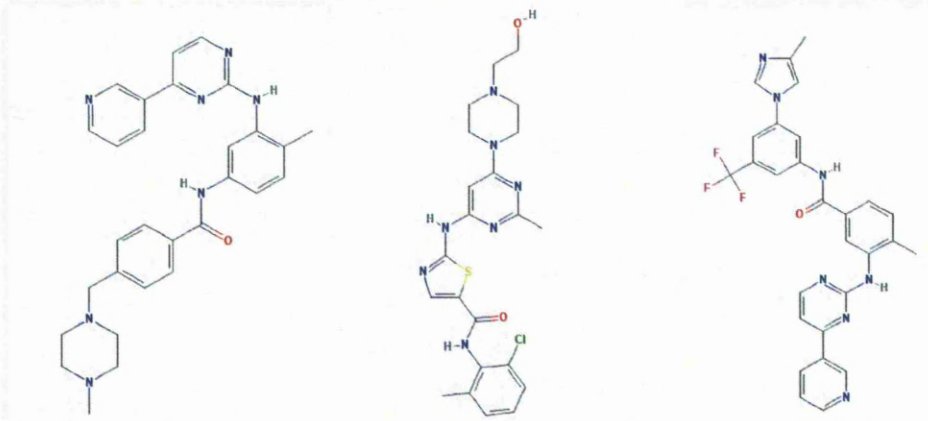
1.5.8. Dasatinib

Dasatinib is a second generation, oral, multi-target inhibitor of BCR-ABL1 and SRC family kinases, licensed by Bristol-Myers Squibb for the treatment of newly diagnosed and imatinib resistant and intolerant CML. The chemical structure of dasatinib is shown in Figure 1.8. *In vitro*, the drug has over 300-fold greater potency than imatinib, and is effective against many BCR-ABL1 kinase domain (KD) mutations that confer imatinib resistance, excluding T315I.[142] Unlike imatinib, dasatinib is able to bind to both the active and inactive conformations of BCR-ABL1.[143] In a phase I study, haematological and cytogenetic responses were observed in both chronic and advanced phase imatinib resistant patients.[144] In a phase II study of imatinib-resistant or intolerant chronic-phase CML patients, 90% achieved CHR and 52% achieved a major cytogenetic response following eight months of treatment with dasatinib. Importantly responses were observed in patients with KD mutations that confer resistance to imatinib.[145]

The phase III DASISION study compares the effectiveness of dasatinib 100mg against imatinib 400mg. At 12 months the CCR rate for dasatinib was 77% while for imatinib it was 66%. The rate of MMR was higher with dasatinib than with imatinib at 46% and 28% respectively. This suggests that dasatinib is a superior drug when compared with standard imatinib,[146] although it is not yet known whether these superior response rates for dasatinib are maintained at years two and beyond.

Our laboratory has shown that dasatinib transport is independent of human organic cation transporter 1 (hOCT1) unlike imatinib[147] and therefore it may be more effective at suppressing BCR-ABL1 tyrosine kinase activity in patients with low hOCT1 expression/activity.[147] hOCT1 will be discussed in more detail in section 1.6.5.

Figure 1.8. Imatinib, dasatinib and nilotinib chemical structure.



Imatinib (Novartis) **Dasatinib (Bristol-Myers Squibb)** **Nilotinib (Novartis)**

Chemical structure taken from PubChem.

1.5.9. Nilotinib

Nilotinib is a selective inhibitor of BCR-ABL1, and was designed by Novartis using the chemical structure of imatinib as a template. Like imatinib, nilotinib binds to the ATP binding site within BCR-ABL1 suppressing its tyrosine kinase activity.[148] Nilotinib binds to the BCR-ABL1 inactive conformation with a higher affinity than imatinib due to its

enhanced topologic fit to the protein. It is effective against the majority of BCR-ABL1 KD mutations which result in imatinib resistance, again with the exception of T315I.[148] Phase II clinical trials have been encouraging with the added observation that patients treated with nilotinib exhibit less side effects than those observed with imatinib treatment.[149] After 24 months of treatment 44% of imatinib resistant and 51% of imatinib intolerant patients achieved a CCR with nilotinib treatment.[150] Overall the rate of MMR was 32% and 39% at 12 and 18 months respectively.[150] Progression-free survival (PFS) at 24 months was 64% and overall survival (OS) was 87%.[150]

The phase III ENESTnd trial randomised patients into one of three arms; imatinib 400mg once daily, nilotinib 300mg twice daily or 400mg twice daily. The rates of CCR by 12 months were significantly higher for nilotinib (80% for the 300mg dose and 78% for the 400mg dose) than for imatinib (65%). At 12 months, the rates of MMR for nilotinib were 44% for the 300mg dose and 43% for the 400mg dose and 22% for imatinib. At 24 months the rates of MMR for nilotinib were 71% for the 300mg dose and 67% for the 400mg dose compared with 44% for imatinib.[151] These data suggest that nilotinib is a superior drug when compared with standard dose imatinib.[152]

1.6.0. CLINICAL LABORATORY PROGNOSTIC FEATURES

Identification of prognostic features at diagnosis is important for the clinical management of patients. The aim of my thesis is to identify new biomarkers predictive of clinical outcome in CML. In this section I will review the current prognostic indicators and outline those parameters/ biomarkers which I will investigate in more detail within this thesis.

Disease stage at presentation is an important prognostic marker, with patients presenting in chronic phase having a better prognosis than patients who present in accelerated phase or blast crisis.[153,154]

1.6.1. Prognostic scoring methods - pre-imatinib era

1.6.1.1 Sokal score

In the pre-imatinib era Sokal and colleagues[155] developed a prognostic scoring calculation. The Sokal score was derived by studying the clinical outcome of 813 patients, most of whom had received busulphan. It is based on the platelet count, percentage of blast cells, age, and spleen size at diagnosis. The Sokal score stratifies patients into three risk groups; low, intermediate and high. Using the calculation Sokal demonstrated that the 4 year survival was 62%, 43% and 33% for the low, intermediate and high risk group respectively. Sokal later reported that both gender and the haematocrit levels were additional prognostic markers in patients younger than 45

years.[156] Sokal also identified that additional chromosome abnormalities conferred a poor prognosis.[157] Although the Sokal score was a valuable prognostic tool in the pre-imatinib era its ability to reliably predict the clinical outcome of patients treated with a tyrosine kinase inhibitor may be more limited. This will be examined in a population study presented in chapter three.

1.6.1.2. Hasford/Euro score

The Hasford/Euro score is another prognostic scoring method. This was derived from over 1500 CML patients treated with interferon at 12 institutions.[158] As well as the components of the Sokal score, the Hasford score additionally incorporates the percentage of eosinophils and basophils. Using the Hasford score the 5 year survival was 76%, 55% and 25% for the low, intermediate and high risk group respectively.

1.6.2. Prognostic scoring method - imatinib era

1.6.2.1. EUTOS (EUropean Treatment and Outcome Study for CML) score

Both the Sokal and Hasford/Euro score were developed in the pre-imatinib era and so there is a requirement to revisit these baseline scoring methods in the imatinib/TKI era. The ELN established a European registry of CML patients receiving imatinib based therapy. A total of 2060 patients, biased for patients faring poorly, was eligible for treatment response assessment. Of these 1261 (61%) patients progressed within 36 months. This is extremely high and does not represent the normal rate of progression

observed with imatinib treatment (which has previously been reported at 6%).[18,159] The authors used CCR at 18 months to stratify patients as responders and non-responders; however the ELN guidelines state that at 18 months patients who are in CCR and not MMR are sub-optimal responders.[111]

In this study neither the Sokal or Euro score had a significant ability to predict the clinical response of patients which is consistent with the data I present in chapter three.[21] The authors suggest a new simplified scoring method known as the EUTOS score which only includes the basophil count and spleen size at diagnosis.[160]

EUTOS score =7 x Basophils + 4 x Spleen size

> 87 = high risk, ≤ 87 low risk

1.6.3. Chromosome 9 deletion

Deletions of the derivative chromosome 9, especially proximal to breakpoint, occur in 10-15% of patients and it has been suggested that these confer a poorer prognosis for interferon and hydroxycarbamide treated patients.[161] Patients with a deletion in chromosome 9 had a short OS and an increased risk of disease progression, which was shown to be independent of the patient's Sokal or Hasford/Euro score.[85] However, in the imatinib era this may be less significant; the previously reported difference in OS was not

observed in imatinib treated patients,[162] although time to disease progression remains shorter in patients with a deletion in chromosome 9.[162]

1.6.4. Timing of response

The prognostic significance of achieving an early molecular response following first-line imatinib treatment was first reported by our laboratory in 2003.[163] After 4 weeks of imatinib treatment patients with a *BCR-ABL1/ABL1* transcript ratio level less than 50% and following 3 months of treatment less than 10%, had a significantly higher probability of achieving a major cytogenetic response at 6 months and a better PFS.[163]

The prognostic value of an early molecular response was also investigated as part of the IRIS trial.[164] Patients with a *BCR-ABL1/ABL1* transcript ratio less than 10% at 6 months and less than 1% at 12 months had a superior EFS and lower probability of disease progression. The study also demonstrated that patients who achieved an MMR by 18 months would not progress to accelerated phase or blast crisis over a five year period.[164] Patients who achieve an MMR by 18 months have a 3% probability of losing their response compared to 26% for patients who had only achieved a CCR.[164] These studies suggest that an early molecular response is a good prognostic indicator.

1.6.5. Drug transporters

Our laboratory has previously shown that imatinib is a substrate for the uptake transporter hOCT1.[165] In more recent work using clinical samples, our group has shown that low hOCT1 expression may be an important mechanism of imatinib resistance and that patients with higher levels of hOCT1 had a greater probability of achieving a CCR, as well as superior PFS and OS.[166] Regression analysis of hOCT1 expression in relation to other parameters such as white blood cell count, platelet count, spleen size at original diagnosis, age, haemoglobin concentration, and phase of disease at commencement of imatinib, together with time since diagnosis until commencement of imatinib were investigated and hOCT1 expression at diagnosis was found to be superior in predicting CCR.[166] However, White *et al*[167] reported that hOCT1 mRNA expression in their cohort of patients did not correlate with CCR. Instead the authors reported that hOCT1 activity (determined by assessing the amount of uptake of C^{14} labelled imatinib that is blocked by prazosin, an inhibitor of hOCT1) did correlate with the clinical outcome of patients, and also correlated with the degree of *in vitro* kinase inhibition (pCrkL/CrkL ratio).[167] Patients with high hOCT1 activity had an 85% chance of achieving a MMR at 24 months compared to 45% of patients with low hOCT1 activity. Low hOCT1 activity can be overcome to a certain extent by using high doses of imatinib thus hOCT1 activity may be useful in identifying patients who may benefit from a higher dose of imatinib or an alternative TKI. Since higher doses of imatinib can overcome low hOCT1 activity, these data may suggest that imatinib is transported by other transporters in addition to hOCT1. hOCT1 activity was found to correlate with PFS and OS, and was not correlated with either the Sokal or Hasford scores.[164,167]

The current literature suggests that hOCT1 is the dominant transporter controlling intracellular imatinib concentration. However, some of the pharmacological transporter inhibitors used[147,167] can affect other transporters and thus other transporters may be important in affecting the intracellular concentration of imatinib.

P-glycoprotein is an efflux transporter which has been shown to confer resistance to treatment in a variety of cancers, by effluxing many chemotherapy agents.[168-170] It is encoded by the ABCB1 (MDR1) gene. High expression of the ABCB1 gene may be associated with imatinib resistance in CML cell lines,[170] and silencing of ABCB1 expression increases the intracellular concentration of imatinib.[171] In contrast, the pre-treatment expression level of the efflux transporters ABCB1, ABCC1 (MRP-1) and ABCG2 has been shown to be unrelated to clinical outcome, though this is controversial.[172]

1.6.6. Plasma imatinib monitoring

Drug compliance, metabolism, and interactions with other drugs are all factors which could influence a patient's response to imatinib treatment. These factors will all affect the intracellular concentration of the drug. Measuring the plasma level of imatinib may provide additional clinical information, especially for patients responding sub-optimally or who have failed imatinib treatment. Picard *et al*[173] reported that a trough plasma level of 1002ng/ml or higher following 12 months of treatment was associated with

achievement of CCR.[173] Plasma monitoring was also performed as part of the IRIS trial, finding that plasma levels of imatinib at day 29 were linked to achievement of CCR. Patients with a trough plasma level of 1009ng/ml or greater had a significantly higher probability of achieving CCR and MMR at 12 months.[174] Additionally patients with higher trough plasma levels had superior EFS. The authors of both studies suggest that plasma monitoring in suboptimal responders could be used to determine compliance to therapy or suspicion of drug-drug interaction, and knowledge of the plasma level may improve the chance of treatment success.[173,174] Conversely, two independent studies carried out in our laboratory[175] and in Canada[176] found no correlation between plasma levels of imatinib and clinical outcome. Plasma monitoring may not therefore be a good deterministic biomarker of clinical outcome in an individual patient, as it is dependent on the timing of the imatinib dose prior to sampling and can be altered by co-medications. Caution is therefore needed when interpreting imatinib plasma level data.

1.6.7. Imatinib resistance and BCR-ABL1 kinase domain mutations

With increasing clinical experience it is becoming clear that there are distinct subgroups of patients who are either primarily resistant or acquire secondary resistance to imatinib.[177] Many cases of acquired imatinib resistance are associated with the emergence of mutations within the BCR-ABL1 KD.[33,178] These are less common in primary imatinib resistance. However, some patients may acquire resistance without KD mutations, whilst others develop KD mutations without developing imatinib resistance,[179] suggesting that additional factors are required to produce a fully drug resistant phenotype.

Currently over 100 BCR-ABL1 KD mutations have been identified. Some mutations remain sensitive to imatinib while others confer resistance (the degree of resistance can also vary between each mutation).[180] Some but not all mutations within the BCR-ABL1 KD impair drug binding to BCR-ABL1 and hence impair the suppression of BCR-ABL1 tyrosine kinase activity.[181,182]

Mutations can be classed into three groups. Firstly, there are those which involve important drug contact sites such as T315I. The second group of mutations are located within the ATP binding loop and confer resistance by preventing ABL1 from adopting the specific conformation required for high-affinity imatinib binding. The third group of mutations are located within the activation loop. These can prevent BCR-ABL1 from adopting the inactive conformation and hence inhibit both imatinib and nilotinib binding. However, dasatinib is able to bind to both the active and inactive conformation and so may still be effective in this subgroup of patients.[183] Common resistant mutations include: for imatinib T315I, M244V, L248, G250E, Y252F,[184] and E255K/V, for dasatinib T315I and F317L[185] and for nilotinib T315I, E255K/V and Y253H.[111,148]

In an Argentinean multi-centre study, mutations were detected in 23% of patients with who failed or lost a response on imatinib treatment. Detection of mutations correlated significantly with accelerated phase, lack of molecular response, and lower cytogenetic and haematological responses, while no difference in OS was observed.[186]

Branford et al[187] reported that a 2-fold or greater rise in BCR-ABL1 transcript ratio was suggestive of a mutation. However, in 2006 our laboratory reported that two consecutive rises in *BCR-ABL1/GUS* transcript ratio could identify patients likely to have a BCR-ABL1 KD mutation and that a single 2-fold or greater rise in *BCR-ABL1/ABL1* transcript did not predict a mutation.[188] These differences are likely to be due to variation in the methodology for measuring the BCR-ABL1 transcript ratio between different labs, e.g. the choice of control gene. Early detection of mutations may be clinically beneficial by allowing early intervention such as switching to a second generation TKI. Many of the data on sensitivity of a specific mutation to TKIs are based on *in vitro* and not *in vivo* data; thus caution is required when interpreting mutation data.[183]

1.6.8. CrkL a surrogate marker of BCR-ABL1 tyrosine kinase activity

It is difficult to accurately assess BCR-ABL1 tyrosine kinase activity directly in primary CML cells due to granulocytic activity which degrades the BCR-ABL1 protein upon lysis.[189] CrkL (CT10, regulator of kinase-like) is an adaptor protein.[190] The normal cellular function of CrkL is to mediate signal transduction through membrane-bound receptors for extracellular matrix, growth factors, and cytokines.[81] CrkL is immediately downstream of BCR-ABL1 and functions to mediate BCR-ABL1 signal transduction.[79-81] CrkL is located centromeric to the BCR gene on chromosome 22 and encodes a 39kDa protein.[191] CrkL consists only of one SH2 and two SH3 domains.[192] The direct interaction of CrkL and BCR-ABL1 is, at least in part, mediated by a proline-rich region in the C-terminal tail of the ABL1 kinase which is localised near the nuclear localisation signal of the ABL1 sequence and binds to the first SH3 domain of CrkL.[81] Studies have shown

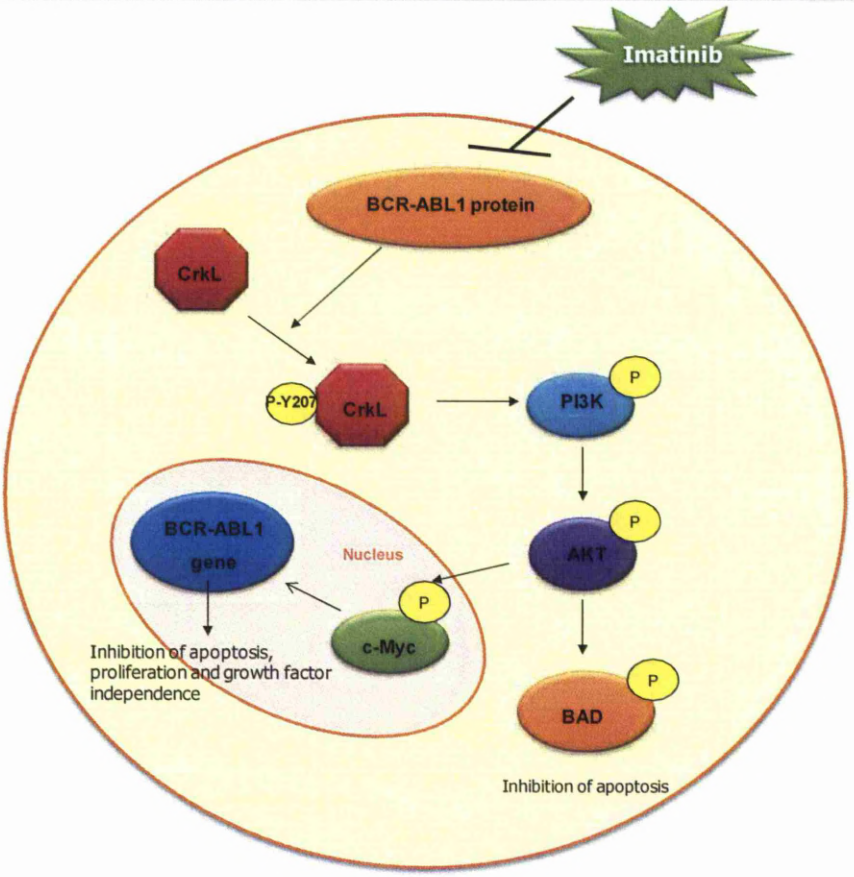
that overexpression of CrkL alone allows cellular transformation to occur.[193-195] Overexpression of CrkL in transgenic mice expressing the p190 BCR-ABL1 protein resulted in the more rapid development of leukaemia.[196] BCR-ABL1 mutants lacking the SH3 binding domain still have the ability to transform myeloid cells suggesting that CrkL remains associated with BCR-ABL1 via its interactions with other proteins.

Phosphorylation of CrkL occurs in the cells of CML patients as a direct consequence of BCR-ABL1, and levels of phosphorylated CrkL correlate with BCR-ABL1 protein levels.[197,198] CrkL phosphorylation status has therefore been identified as a surrogate marker for BCR-ABL1 tyrosine kinase activity (Figure 1.9).[197,199]

Immunohistochemistry has demonstrated that pCrkL levels are elevated in the following malignancies;[200] breast cancer (49%), lung carcinomas (55%), skin cancer (67%), ovarian cancer (50%) and colon carcinomas (63%). Recently CrkL mRNA was found to be over-expressed in Non-Small Cell Lung Cancer (NSCLC). Knockdown of CrkL in lung cancer cell lines led to significantly decreased cell proliferation, cell cycle progression, cell survival, invasion and cell motility, suggesting a significant role for CrkL in NSCLC.[201]

Deletion of the CrkL gene on chromosome 22 causes DiGeorge syndrome, a condition with a variety of clinical features including congenital heart disease, facial disfiguration, hypoparathyroidism and increased risk of schizophrenia.[202]

Figure 1.9. BCR-ABL1 and CrkL signalling pathway.



1.6.8.1. Current approaches for detecting and assessing CrkL phosphorylation status.

White *et al*[197] investigated the imatinib induced *in vitro* inhibition of pCrkL and CrkL levels by Western blotting in 60 patients. Using Western blotting and densitometry of pCrkL and CrkL protein bands they produced a ratio, which was then expressed as a

percentage against normalised values. They reported that imatinib IC50 (defined as the imatinib concentration that reduces the pCrkL/CrkL ratio by 50%) was predictive of subsequent clinical outcome of imatinib treated newly diagnosed CP patients. However, this is of limited practical use since Western blotting can be time consuming and also requires a large number of cells (>10^6) which may be difficult to obtain from CML patient samples, especially once treatment has begun. In a subsequent paper White *et al*[198] measured pCrkL/CrkL levels following two or three weeks of *in vivo* imatinib treatment and found that this ratio also correlated with clinical outcome.

In 2006 Hamilton *et al*[199] developed a method for the detection of pCrkL by flow cytometry. Flow cytometry has the advantage that it requires fewer patient cells (~ 10^3 cells), produces quantitative values, and results can be obtained rapidly. They demonstrated that flow cytometry results were comparable to those produced by Western blotting. Unphosphorylated CrkL was not assessed, so it was not possible to calculate a pCrkL/CrkL ratio as suggested by White *et al.*[197] Furthermore, their study was limited to previously frozen CD34+ cells and the relationship with clinical outcome was not investigated. More recently Khorashad *et al*[203] investigated the relationship between pCrkL levels and clinical outcome using previously frozen CD34+ cells from 36 newly diagnosed CML patients and found no correlation with clinical outcome.

The first biomarker to be investigated in this thesis is pCrkL and CrkL as a surrogate marker for BCR-ABL1 tyrosine kinase activity. This involves optimising reported

techniques and developing a FACS based assay for rapid screening of clinical samples to investigate the predictive value of pCrkL and CrkL in newly diagnosed CML patients. These data will be presented in chapter four.

1.6.9. Predicting disease progression

Mechanisms underlying the evolution of blast crisis are poorly understood. It is thought that disease progression is a multi-factorial process initiated by both BCR-ABL1 dependent and independent mechanisms.[204] Genetic lesions such as additional chromosomes, deletions, insertions or mutations of genes (including BCR-ABL1 itself e.g. T3151) occur as patients progress to blast crisis.

Currently there are no biomarkers which can predict at diagnosis those patients who will progress into blast crisis. This will be explored in more detail in chapters seven and eight.

1.6.9.1. Microarray analysis

Gene expression profiles may be helpful in patient prognostication. However, most methodologies are time consuming and result in a large list of candidate genes and, most importantly, these techniques cannot be easily adapted for routine clinical use. Using the Bayesian model averaging (BMA) method on a large microarray data set Radich *et al*[205] identified six genes (NOB1, DDX47, IGSF2, BLT1 and SCARB1 and SLC25A3) that

discriminate between early chronic phase, late chronic phase, accelerated phase and blast crisis.

Yong *et al*[206] performed a gene expression profiling study using diagnostic CD34+ cells from patients who either progressed to blast crisis or remained well in CP. A multivariate Cox regression model identified the combination of low CD7 expression with high expression of proteinase 3 or elastase as associated with longer survival. The potential application for this assay would be to identify at diagnosis those patients at a greater risk of disease progression. However, this approach has yet to be tested prospectively.

Certain genes have been identified as being altered depending on whether the patient has lymphoid or myeloid blast crisis. Myeloid blast crisis is associated with changes in both p53.[207,208] and the transcription factor RUNX1.[83] p53 is a tumour suppressor and is inactive in 30% of solid tumours. During progression to blast crisis deletions and rearrangements of the p53 gene have been identified.[209] The transcription factor Ikaros[210] and CDKN2A/B[211] (Cyclin-dependent kinase inhibitor 2A/B) are associated with lymphoid blast crisis. EVI-1[212] and WT1[213] have also been reported to play a role in blast crisis. These genetic changes occur as patients progress into blast crisis but although they are associated with disease progression, they offer no prospective prognostic information. The EVI-1 oncogene has been reported to be up-regulated during blast crisis. Detection of EVI-1 mRNA in imatinib failure patients correlates with a poorer EFS, PFS and OS when the patients are treated with a second generation TKI. Screening for EVI-1

expression at the time of imatinib failure may predict for response to second generation TKI.[212]

BCR-ABL1 via its tyrosine kinase activity can also cause post-translational modifications of the following proteins altering their activity; c-Myc[94,214,215] JAK2,[216] Lyn,[216,217] hnRNP-E2,[218] STAT5,[68,168,219] BMI-1,[220] and BCL-2, all of which are altered at disease progression.[204]

1.6.9.2. TWIST1

TWIST1 is a transcription factor involved in embryogenesis, cell proliferation and cell survival.[221-223] A small study of CML patients reported that *TWIST1* mRNA expression was up-regulated in CD34+ cells from imatinib resistant patients as well as during disease progression. Furthermore, *TWIST1* was found to be over-expressed in CML diagnostic samples of patients who later developed resistance to imatinib.[223] *TWIST1* may be a biomarker of imatinib resistance, although this needs to be confirmed on a larger cohort of patients.

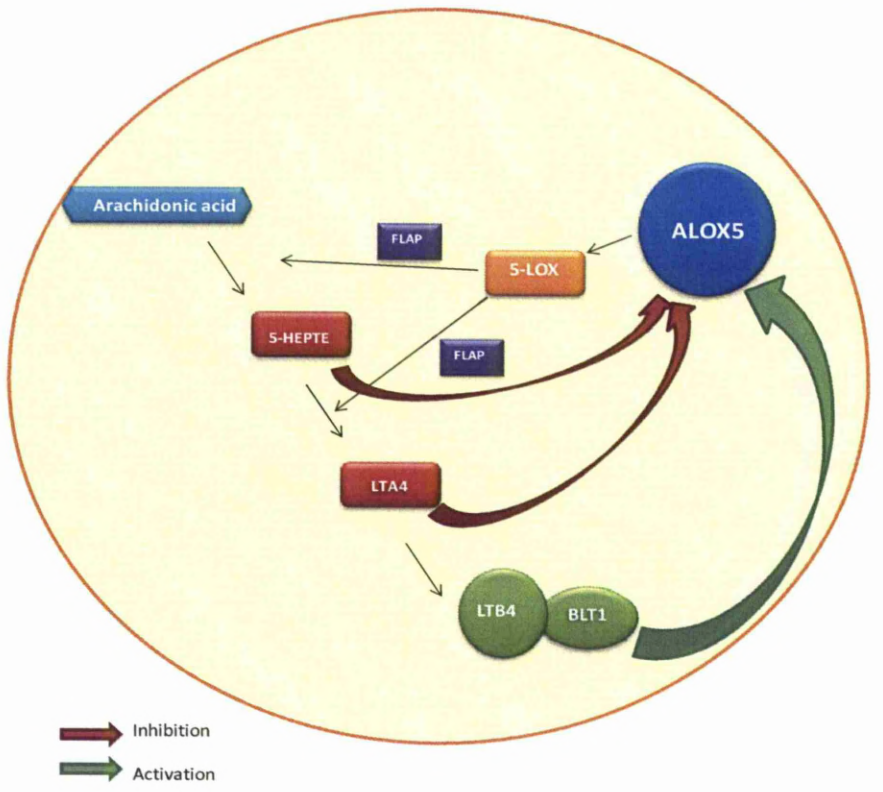
1.7.0. LEUKAEMIC STEM CELLS AND *ALOX5*

Most malignancies are initiated by a small population of cells known as cancer stem cells; thus these are the ultimate therapeutic target. Leukaemic stem cells (LSC) are believed to be a population of cells required for the initiation and maintenance of the disease.[224-226] Therapeutically the difficulty lies in the identification of differences between normal and leukaemic stem cells, in order to ensure that the leukaemic stem cells are selectively targeted. Genes such as Wnt/β- catenin,[227-229] Hedgehog,[230] Notch,[231] Bim1[127] and p53[209] have been identified as playing an important role in cancer development; however all these genes also play an active role in normal stem cells.

1.7.1. *ALOX5*

The *ALOX5* (Arachidonate 5-lipoxygenase) gene encodes a member of the lipoxygenase gene family and plays a role in the synthesis of leukotrienes from arachidonic acid. The encoded protein is expressed specifically in bone marrow-derived cells, and catalyses the conversion of arachidonic acid to 5(S)-hydroperoxy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-HEPTE), and further to the allylic epoxide 5(S)-trans-7,9-trans-11,14-cis-eicosatetraenoic acid (leukotriene A4/LTA4). LTA4 is unstable and is converted to LTB4 which is more stable. 5-HEPTE and LTA4 negatively regulate *ALOX5* expression; positive regulation of *ALOX5* occurs when LTB4 binds to its receptor BLT1 (Figure 1.10).[226,232-236]

Figure 1.10. Arachidonic acid metabolism pathway.
5-LOX is encoded by the *ALOX5* gene and is activated by the enzyme FLAP. Arachidonic acid is converted to 5-HEPTE by 5-LOX which is further converted to LTA4 by 5-LOX. Both 5-HEPTE and LTA4 negatively regulate *ALOX5* expression. LTA4 is unstable and is further converted to LTB4. The binding of LTB4 to its receptor BLT1 positively regulates *ALOX5* expression.



1.7.2. Leukotriene B4 (LTB4)

Leukotrienes are a family of pro-inflamatory lipid mediators which play an important role in inflammation. Leukotrienes are generated in leukocytes through the metabolism of arachidonic acid via the 5-lipooxygenase pathway.[237] LTB4 activates leukocytes on the

endothelium allowing them to migrate into tissues. In neutrophils, LTB4 is a chemo-attractant and can induce the formation of ROS.[238]

1.7.3. LTB4 receptor – BLT1

LTB4 has two G protein coupled receptors, BLT1 and BLT2. BLT1 is a high affinity receptor for LTB4 and is only expressed on leukocytes, while BLT2 is a low affinity receptor expressed on leukocytes, spleen, liver and ovarian tissue.[239] The classical function of BLT1 is to mediate LTB4 induced neutrophil and macrophage migration to sites of inflammation via chemotaxis and up-regulation of adhesion molecules.[240] BLT1 is up-regulated in rheumatoid arthritis and chronic obstructive pulmonary disease, thus BLT1 antagonists are currently being explored as therapeutic options in these conditions.[241] LTB4 must bind to BLT1 to mediate its positive feedback on *ALOX5*.

1.7.4. The role of *ALOX5* role in asthma and coronary heart disease

Leukotrienes are known to cause symptoms in asthma, causing smooth muscle contraction, impairing mucociliary clearance, enhancing mucus secretion and attracting eosinophils to the airways.[234,242,243] Zileuton, an *ALOX5* inhibitor, has been developed for the treatment of asthma as an alternative to low-dose inhaled glucocorticosteroids.[244] *ALOX5* is also a target for pharmaceutical intervention in coronary heart disease. Mutations in the promoter region of this gene lead to a diminished response to anti-

leukotriene drugs used in the treatment of asthma and may also be associated with atherosclerosis [235,243,245]

1.7.5. *ALOX5* and Cancer

Alterations in the *ALOX5* pathway can result in increased cellular proliferation, survival, and suppression of apoptosis in human cells. It thereby plays a significant role in human carcinogenesis. ALOX5 protein and mRNA expression were found to be elevated in 79% of oesophageal cancer tissue compared to normal oesophageal mucosa.[236] The *ALOX5* gene is up-regulated in pancreatic cancer.[246,247] Furthermore, the *ALOX5* metabolite 5(S)-HEPTE has been shown to stimulate cellular proliferation via the activation of the mitogenic intracellular tyrosine kinases MEK/ERK and PI3 kinase/AKT. In colorectal cancer, exposure to cigarette smoke extract has been shown to enhance ALOX5 protein expression in inflammation-associated colonic adenomas. This effect is mediated in part by deregulated ALOX5 DNA demethylation, and up-regulation of matrix metalloproteinase-2 (MMP-2) and vascular endothelial growth factor (VEGF), all key angiogenic factors for tumourigenesis. These effects can be reversed by treating colon cancer cells with dual *ALOX5* and COX-2 inhibitors.[248] In patients with ovarian cancer *ALOX5* expression correlated with OS, and single nucleotide polymorphisms (SNPs) in the *ALOX5* gene were identified in patients with a poorer outcome.[249]

1.7.6. *ALOX5* and CML

CML leukaemic stem cells are not sensitive to imatinib or second generation tyrosine kinase inhibitors which are the current treatment option for CML patients.[250-252] Chen *et al*[253] investigated changes in mouse leukaemia stem cells (LSC) genes pre and post imatinib treatment using DNA microarray analysis. *ALOX5* was up-regulated in LSC, and the up-regulation was not inhibited by imatinib treatment. Furthermore, mice transplanted with BCR-ABL1 positive bone marrow which was *ALOX5* deficient were resistant to the induction of CML. Additionally, *ALOX5* deficiency had no effect on the growth of BCR-ABL1 negative cells, suggesting that *ALOX5* is important for malignant LSC but not for normal haematopoietic stem cells. These data suggest that *ALOX5* is essential for the development of CML.[253] *ALOX5* can be inhibited by the 5-lipoxygenase inhibitor zileuton, and knocking down of *ALOX5* results in decreased β-catenin expression.[253] *ALOX5* function can be assessed by measuring the concentration of the stable leukotriene LTB4 in plasma.[253] LTB4, the product of the 5-LO pathway, was found to be elevated in a mouse model of CML.

In summary, *ALOX5* expression and function are associated with prognosis in solid tumours and the Chen *et al* paper[253] suggests that *ALOX5* is essential for the development of CML in mice. The aim of chapter six in the current thesis is therefore to investigate the role of *ALOX5* in clinical CML samples, and to determine if the level of *ALOX5* can predict a patient's clinical outcome.

1.8.0. THE ROLE OF PROTEIN PHOSPHATASE 2A (PP2A) IN CML

1.8.1. PP2A structure and biology

Many cells utilise reversible phosphorylation as a mechanism of post-translational modification for activating and deactivating key regulatory molecules involved in cell signalling.[254] Deregulation of reversible phosphorylation can have a significant impact on cellular behaviour. Alterations in the action of kinases and phosphatases give rise to many forms of cancer.[12,255]

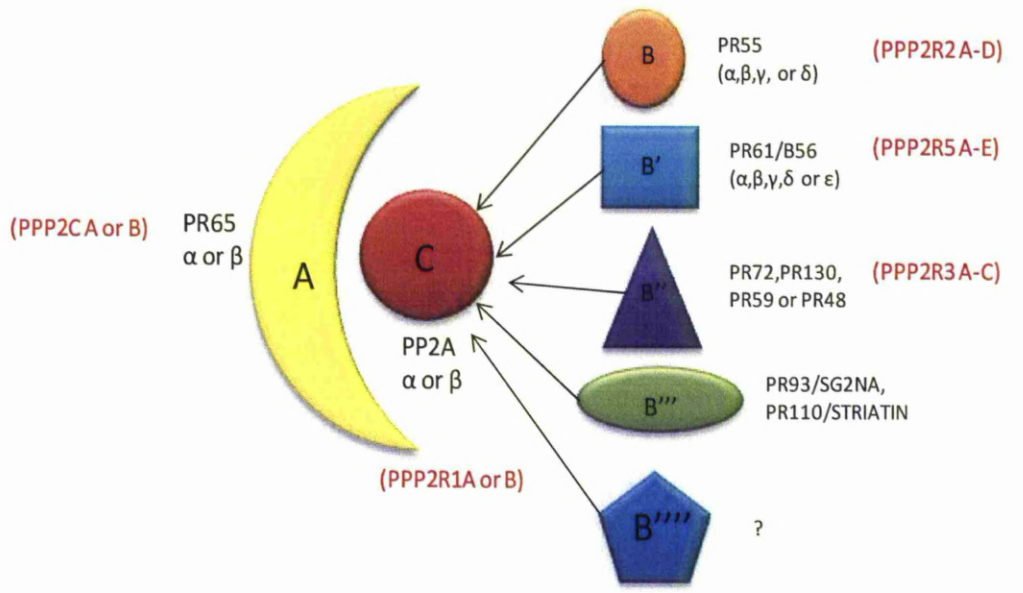
A major cellular serine/threonine phosphatase working in opposition to kinases is the tumour suppressor Protein Phosphatase 2A (PP2A).[254] PP2A plays an important role in regulating cell proliferation, differentiation and survival. Loss of PP2A function has been associated with cellular transformation; thus it is also known as a tumour suppressor.[256] PP2A is highly conserved from yeast to humans and accounts for 1% of total cellular proteins. Along with PP1 (Protein Phosphatase 1), PP2A accounts for 90% of the serine/threonine phosphatase activity in a cell (Figure 1.12).

1.8.1.1. Catalytic subunit – PP2Ac

The PP2A catalytic subunit (PP2Ac) targets phosphate groups on either serine or threonine residues. Two mammalian isoforms of PP2Ac have been identified, named α and β, which are 97% identical in primary sequence but are encoded by different genes.

PP2Acα is expressed 10 fold more than β and the PP2Acα promoter is 7-10 times more active than β. Expression of PP2Ac is tightly controlled to ensure a constant level of PP2A. Activity of PP2A is controlled by post-translation modification. Loss of PP2Acα is lethal in yeast, demonstrating the importance of maintaining cellular homeostasis.[257]

Figure 1.12. PP2A protein structure.
Diagram of the PP2A trimeric complex, illustrating the scaffolding subunit A binding to the catalytic subunit C to mediate the binding of the B subunits. B subunits interact via the same or overlapping sites within subunit A of the core dimer to form an active PP2A protein. (Adapted from Eichhorn *et al*[257]).



1.8.1.2. Regulatory subunit A subunit (PR65) - PPP2C A or B

The scaffolding subunit A (PR65) binds to the catalytic subunit C to mediate the binding of the B subunits. Two isoforms of the scaffolding subunit A have been identified, named PR65α and PR65β. Both isoforms are ubiquitously expressed, though PR65α is expressed at a higher level than PR65β. PR65β has been identified as a tumour suppressor, as mutations in PR65β are associated with lung and colon cancer.[254]

1.8.1.3. Regulatory B subunits

B subunits determine tissue specificity and sub-cellular localisation of the PP2A protein. Details about the different B subunits can be found in Table 1.2.

Table 1.2. Characteristics of B subunits.
B subunits determine tissue specificity and sub-cellular localisation of the protein.

B Subunits	Details
B or PR55 family - PPP2R2 A-D	• 55kDa encoded by 4 genes PR55 α,β,γ,δ (A-D). • Expression is tissue specific. • α δ are widespread, β,γ limited to brain.
B' or PR61 family (PPP2R5 A-E)	• encoded by 5 genes PR61 α,β,γ,δ ε. • human B' β has two isoforms – β1 and β2. • B' γ has three isoforms – γ1, γ2 and γ3. • All B' isoforms are 80% identical.
B'' or PR72 family - (PPP2R3 A-C)	• Mainly found in heart and skeletal muscle. • Found in all tissues but at higher concentration in heart and skeletal muscle.
B''' or PR93/PR110 family	• Function as scaffolding proteins. • Involved in Ca^{2+} dependent signaling.

1.8.2. Regulation of PP2A

PP2A is regulated by post-translational modifications such as methylation and phosphorylation. Phosphorylation occurs on tyrosine 307, which is located in the conserved C-terminal part of PP2Ac, and prevents the recruitment of B subunits, resulting in inactivation of the enzyme.[254] Phosphorylation can also occur on threonine 304; however the functional significance of phosphorylation at this site is unclear.[257] PP2A can rapidly re-activate itself in an auto-dephosphorylation reaction. Tyrosine phosphorylation of PP2Ac is enhanced in the presence of the phosphatase inhibitor okadaic acid (OA).[254]

Methylation of PP2Ac varies during the cell cycle. The mechanism and the physiological consequences of this oscillating methylation are unknown. Conflicting data exist with regard to the effect of PP2Ac methylation on its catalytic activity, with one group observing a moderate increase,[258] another seeing no effect,[259] and a third observing a decrease in phosphatase activity.[260] Methylation of the C terminal tail by leucine carboxyl transferase 1 (LCMT1) is required for the binding of the PR55 B subunit but not the other B subunits and this can be reversed by phosphatase methylesterase (PME-1).[261]

1.8.3. PP2A and CML

PP2A is inactive or mutated in many solid tumours[93,262,263] and haematological malignancies.[264,265] In CML cells PP2A is a key target of BCR-ABL1 signalling; this

protein becomes inactivated in these cells because BCR-ABL1 prevents the auto-dephosphorylation at tyrosine 307.[264,266] Maintenance of pY^{307}-PP2A levels in CML cells feeds back to BCR-ABL1 and facilitates increased and sustained kinase activity. Inhibition of BCR-ABL1 by imatinib results in reactivation of PP2A, inducing both suppression of growth and enhanced apoptosis of the leukaemic cells.[264] However, it is unknown whether Y^{307} in PP2A is a direct substrate of BCR-ABL1, and the mechanism regulating phosphorylation of PP2A at this site is not clearly defined.

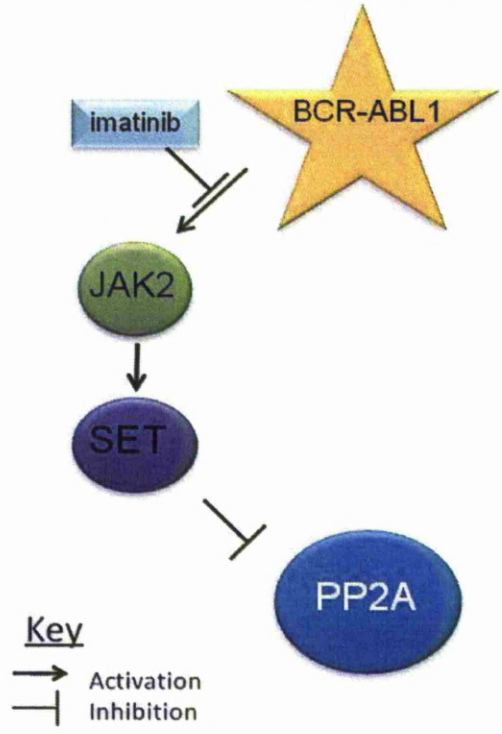
BCR-ABL1 might regulate PP2A activity in CML cells via the PP2A inhibitor protein SET. In CML cell lines, increased expression of BCR-ABL1 increases expression of SET through a process that is mediated by active JAK2 (Figure 1.13).[216,264] Furthermore, Neviani *et al*[264] also observed that SET levels rose at evolution of blast crisis in two cases of paired chronic phase and blast crisis primary CML samples, and that incubation with imatinib decreased SET levels in primary CML cells from a single patient undergoing blast crisis.

1.8.4. PP2A inhibitor - SET

The *SET* gene is located on chromosome 9q34.11, centromeric to c-abl[267] and gives rise to a putative 39kDa protein that shuttles between the nucleus and cytoplasm under the influence of hnRNP A1.[268] It has also been reported to be over-expressed in solid tumours

as well as leukaemia.[264,269,270] The normal physiological function of SET is not fully understood.[271]

Figure 1.13. Model mechanisms by which SET/JAK2 regulates PP2A and BCR-ABL1 signalling.
Possible mechanism of PP2A inhibition in CML. BCR-ABL1 up-regulates JAK2 which in turn increases the expression of the SET leading to the inhibition of PP2A. Imatinib can reactivate PP2A by decreasing both JAK2 and SET.



1.8.5. PP2A inhibitor - *SET binding protein 1 (SETBP1)*

SET binding protein (SETBP1) protects SET from protease cleavage leading to an increase in the levels of full-length SET and formation of a SETBP1-SET-PP2A complex, which in turn results in the inhibition of PP2A phosphatase activity. Recently Cristobal *et al*[265] reported that overexpression of *SETBP1* mRNA predicts adverse outcome in elderly patients with acute myeloid leukaemia (AML) and SETBP1 promotes the proliferation and expansion of leukaemic cells.[265] However, the role of *SETBP1* in CML is unknown and warrants further investigation since SET has been reported to inhibit PP2A CML.

The role and predictive value of measuring PP2A, inactive PP2A and its two inhibitors will be addressed in chapters seven.

1.8.6. Cancerous Inhibitor of PP2A (CIP2A)

Cancerous Inhibitor of PP2A (CIP2A, also known as "constans interacting protein 2A" and KIAA1524) has recently been described as a novel inhibitor of PP2A.[271] In a cancer setting CIP2A functions by preventing PP2A driven dephosphorylation of pS^{62}-c-Myc, which results in greater stabilisation of c-Myc protein as demonstrated by a longer half-life, as well as reduced proteolytic degradation. c-Myc can mediate genetic instability resulting in tetraploidy and aneuploidy.[96,97] CIP2A has no normal cellular function; it is

not essential for cell cycle progression or cell viability but functions to propagate malignant cell growth via its stabilisation of c-Myc.[271]

In gastric cancer, CIP2A mRNA was found to be over-expressed in 87% of tumour samples. Immunohistochemical analysis revealed that CIP2A protein was readily detectable in malignant samples but not in normal gastric mucosa. CIP2A depletion in the AGS gastric cancer cell line resulted in inhibition of growth and clonogenic capabilities as well as reduced stability and expression of c-Myc protein.[91,272] Furthermore, CIP2A protein was associated with a poorer OS in patients with tumours less than 5cm, with the 10 year overall survival for CIP2A positive patients being 8.1% compared to 37.6% in the CIP2A-negative group. CIP2A may therefore be an adverse prognostic feature in gastric cancer.[91]

Similarly in breast cancer, CIP2A mRNA was found to be over-expressed in 39% of samples and was associated with the aggressiveness of the disease as well as promoting the *in vitro* malignant growth of breast cancer cells. [93]

CIP2A depletion triggered partial differentiation of the acute promyelocytic leukaemia cell line HL60[272] and decreased growth of gastric[91] and breast cancer cells.[93] In mouse neural progenitor cells, overexpression of CIP2A increases progenitor cell self-renewal and proliferation, and both c-Myc and CIP2A enhance each others expression.[273] At the

time of conducting this work no studies had investigated the role of CIP2A in any haematological malignancy. This novel PP2A inhibitor therefore warrants investigation in CML; this will be addressed in chapter eight.

1.8.7. Proviral Integrations of Moloney virus (PIM1)

PIM1 (Proviral Integrations of Moloney virus 1) is a constitutively active serine/threonine kinase, thus its activity is dependent on the absolute amount of protein.[274] PIM kinases are mediators of cytokine signalling pathways in haemopoietic cells and contribute to the progression of certain types of leukaemia and solid tumour.[274] Overexpression of PIM1 in transgenic mice resulted in lymphoma, leading to PIM1 being classified as a proto-oncogene.[255,275] Co-expression of PIM1 and c-Myc in transgenic mice is embryonically lethal due to the formation of lymphomas *in utero*.[92] These data suggest that although PIM1 is a proto-oncogene in its own right, its effects are mild. However, when expressed with the oncogene c-Myc, a lethal synergistic transformation effect can occur.[92] PIM1 can phosphorylate and stabilise c-Myc. PP2A can inactivate PIM1 and target it for degradation, thus levels of PIM1 are indicative of PP2A activity and function.[276]

In prostate cancer both c-Myc and PIM1 are over-expressed, and PIM1 overexpression increases the tumourigenicity of prostate cancer cell lines.[92,274,277] The exact molecular mechanism involving PIM1 and c-Myc interaction is not fully understood. It is thought that PIM1 may phosphorylate c-Myc, thus preventing it from being degraded.

PIM1 is important for the survival of BCR-ABL1 positive cell lines and it is thought that PIM1 may play an anti-apoptotic role, as well as mediating proliferation.[278] PIM1 protein is up-regulated in a BCR-ABL1 kinase-dependent manner in CD34+ cells when comparing CP with BC samples. This up-regulation is associated with expression of microRNA miR328.[218]

A previous report[91] regarding CIP2A has suggested that it functions by inhibiting the destruction of c-Myc, and this in part can be mediated via PIM1. While investigating the role of CIP2A in CML, the role of c-Myc and PIM1 will also be explored, in chapter eight.

1.9.0. AIMS OF THIS THESIS

Although imatinib has dramatically altered the outlook for CML, a minority of patients still fare badly. The prime aim of this thesis is to investigate whether patients destined to respond poorly to imatinib can be identified prospectively. This would be clinically useful as they might then be candidates for alternative treatment strategies, such as early SCT or a second generation TKI from initial diagnosis.

The aims of this work are:

1. To establish if IRIS and other clinical trial data can be extrapolated to an unselected CML population. This will be done by determining 'real' rates of CCR and MMR, and thus the rate of imatinib failure, for patients treated within our region.

2. To evaluate recently reported biomarkers for CML outcome for their significance in determining outcome of modern CML therapy:

    a. Whether BCR-ABL1 tyrosine kinase activity can predict outcome.

    b. Whether the *BCR-ABL1* transcript type affects outcome.

3. To investigate new biomarkers for their predictive value on outcome.

    a. Investigate the role of *ALOX5* in human CML for the first time.

b. Investigate the role of PP2A and its inhibitory proteins (SET, SETBP1 and CIP2A) in CML cells and ascertain whether any /all are predictive of clinical outcome.

CHAPTER TWO - Patients and methods

2.1.0. PATIENTS

In our area of the North West of England, the adjacent North Wales coastal strip and the Isle of Man (total population 2 million), all services for adults with haematological cancer are located at 12 hospitals. Molecular diagnosis of CML and monitoring for *BCR-ABL1* transcripts are carried out at a single centre (Royal Liverpool University Hospital) for all CML patients in this geographical area. The clinical outcome and molecular data of all patients in our area are documented at our centre. Similarly, conventional G-banded metaphase analysis for t(9,22) and additional chromosome lesions, and fluorescent *in situ* hybridisation (FISH) in our geographical area are carried out at one of three regional laboratories (Liverpool, Manchester and Cardiff). Where required, metaphase and FISH data at appropriate time points were obtained from these laboratories.

2.2.0. DEFINING CLINICAL RESPONSE

Patients were stratified into three clinical outcomes:

- **CCRe** - complete cytogenetic response after 12 months of imatinib treatment. This group corresponds to "optimal responders" as defined in the ELN 2009 guidelines.[111]
- **No-CCRe** – Patients who had achieved a complete haematological response but not a complete cytogenetic response after 12 months of imatinib treatment, and who had not progressed. This group corresponds to "sub-optimal responders / failures" as defined in the ELN 2009 guidelines.[111]
- **Blast crisis (BC)** – patients who presented in chronic phase but who subsequently progressed into blast crisis, irrespective of their response at 12 months.

2.3.0. SAMPLE COLLECTION AND PREPARATION

At diagnosis and after 1, 2, 3, 6, 9 and 12 months of imatinib treatment, 20-30mls of peripheral blood was collected into EDTA. The blood was processed according to the requirements of each study.

2.3.1 Total leukocytes

Erythrocytes were depleted using red cell lysis buffer (0.1M ammonium chloride, 10mM sodium bicarbonate and 1.3mM EDTA (Sigma-Aldrich, Dorset, UK). Blood was mixed with lysis buffer for 5 minutes then centrifuged at 770g for 10 minutes, washed with PBS (phosphate buffered saline), centrifuged for a further 5 minutes and then the cells were pelleted. For RNA extraction total leukocytes were resuspended in RLT buffer (Qiagen, West Sussex, UK) containing 1% β-mercaptoethanol (Sigma-Aldrich) and stored at -20°C for extraction at a later time point. Alternatively total leukocytes were used fresh as required.

2.3.2 Mononuclear cells (MNC) preparation

Mononuclear cells (MNC) were prepared from 20-30mls of peripheral blood. MNC were separated by density-dependent centrifugation (Lymphoprep Axis-Shield, Cambridge, UK), washed in RPMI (Roswell Park Memorial Institute) 1640, and resuspended in RPMI 1640 containing 10% dimethyl sulphoxide (DMSO) (Sigma-Aldrich) and 10% foetal calf serum (FCS; BioSera, Ringmer, UK) at 4°C and cryopreserved in liquid

nitrogen. When required, cells were thawed at 37°C and the DMSO was diluted to <1% over a period of 30 minutes via dropwise addition of 10% FCS/RPMI. After washing, cells were re-suspended at 2 x 10^6 /ml in 'culture medium' (RPMI 1640 containing 10% FCS, 1% L-glutamine and 2% penicillin / streptomycin; Invitrogen, Paisley, UK), seeded into 6-well tissue culture plates (Becton Dickinson (BD), Oxford, UK) and cultured under standard tissue culture conditions (5% CO_2 in air, 37°C, 100% humidity) overnight. MNC from normal healthy volunteers were used fresh.

2.3.3 Preparation of CD34+ cells

Samples were enriched for CD34+ cells using the CliniMACS kit (Miltenyi Biotec, California, USA) according to the manufacturer's instructions, then resuspended in 10% DMSO/10% FCS/RPMI at 4°C and cryopreserved in liquid nitrogen. All CD34+ cells were collected at diagnosis and then grouped according to the patients' subsequent clinical outcome as defined in section 2.2: CCRe (n=3), No-CCRe (n=4) or BC (n=3). In some experiments were purified CD34+ cell samples were not available, MNC were co-stained with a CD34 antibody (BD), and FACS analysis was performed gated on CD34+ cells.

2.3.4 Plasma preparation

5ml of peripheral blood was centrifuged at 770g for 15 minutes. Plasma was collected from samples, aliquoted and stored at -20°C prior to use.

2.4.0. GENE EXPRESSION ANALYSIS

2.4.1 RNA extraction

RNA was extracted using RNeasy mini kit (Qiagen). Samples (as prepared in section 2.3.1) were thawed and transferred to a QIA shredder column sitting in a 2ml collection tube, and centrifuged at 14,000g for 2 minutes in a bench-top microcentrifuge. 600µl of 70% ethanol was added and mixed well with the flow through in the 2ml collection tube. 600µl of sample was added to a RNeasy mini spin column sitting in a 2ml collection tube and centrifuged for 15 seconds at 14,000g. The flow through was discarded. 700µl of RW1 buffer was added to the RNeasy column and left at room temperature for 5 minutes, followed by centrifugation at 14,000g for 5 minutes, then the flow through was discarded. The RNeasy spin column was transferred to a new RNeasy collection tube. The RNeasy spin column was washed twice with 500µl RPE buffer and centrifuged at 14,000g for 15 seconds. The RNeasy spin column was transferred into a new 1.5ml collection tube, and then 40µl of RNase free water was added directly onto an RNeasy membrane and left at room temperature for 5 minutes. RNA was eluted by centrifuging for 2 minutes at 14,000g. RNA was stored at -70°C.

2.4.2 cDNA synthesis

cDNA was synthesised using RNA prepared above. 30µl RNA was incubated with 2µl (500ng/µl) of random hexamers (Promega, Southampton, UK) at 70°C for 10 minutes, then cooled on ice for 3 minutes. 16µl 5xRT buffer, 8µl of 0.1M DTT (DL-Dithiothreitol) and 4µl of 10mM dNTP (SuperScript III Reverse Transcriptase kit, Invitrogen) was

added to the sample and incubated for 5 minutes at 25°C. 1µl of 200u/µl Superscript II reverse transcriptase was added to the sample and heated at 25°C for 10 minutes, then at 42°C for 60 minutes and finally at 70°C for 15 minutes to stop the reaction. cDNA was stored at -20°C.

2.4.3 Identification of BCR-ABL1 transcript type.

The *BCR-ABL1* transcript type was identified manually by amplifying cDNA and performing a PCR reaction. Each PCR reaction consisted of 2µl cDNA, 1.5mM Mg_2Cl, 200µM of each dNTP, 0.5µM of each primer (MWG Biotech, Milton Keynes, UK) and 1 unit of GoTaq Flexi DNA polymerase (Promega). Primer sequences are given in Table 2.1. PCR conditions were: 95°C for 10 minutes, then 32 PCR cycles consisting of a denaturation step at 95°C for 1 minute followed by an annealing step at 62°C for 30 seconds and an extension step at 72°C for 1 minute. A final extension step was carried out at 72°C for 10 minutes.

To determine the size of the PCR product, 15µl of PCR product was mixed with 3µl 6x loading buffer and loaded onto a 1% agarose gel and run in 0.5M Tris-Borate-EDTA buffer (Sigma-Aldrich) at 120 volts for 90 minutes. The agarose gel was stained with 0.5mg/ml ethidium bromide buffer for 15 minutes. A photograph was taken under UV light. Three sets of primers (EK158 with EK159, NA4 with B2A, and E1F with NA4) were used to amplify *BCR-ABL1* [279] and the primers NA4 and A2N were used to amplify

ABL1 (Table 2.1). KCL22 and LAMA84 cells lines along with an e1a2 plasmid were used as positive controls.

Table 2.1. BCR-ABL1 manual and real-time PCR primer and probe sequences

Primer name	Sequence
A2N	5'-CCC AAC CTT TTC GTT GCA CTG T-3'
B2A	5'-TTC AGA AGC TTC TCC CTG ACA T-3'
EK158	5'-CAC GTT CCT GAT CTC CTC TGA C-3'
EK159	5'-TCC AAC GAG CGG CTT CAC TCA G-3'
NA4	5'-CGG CTC TCG GAG GAG ACG TAG A-3'
E1F	5' AGA TCT GGC CCA ACG ATG G
BAHP1	5' TGA AAA GCT CCG CTT AGG CTA TAA TCA-Fluoresence
BAHP2	5'LC Red 640-AAT GGG GAA TGG TGT GAA GCC CAA A-P

2.4.4 Quantification of BCR–ABL1 expression by quantitative real-time PCR (qRT-PCR).

qRT-PCR was performed on a LightCycler (Roche Diagnostics, East Sussex, UK). The LightCycler uses two fluorophore-labelled probes that hybridise to adjacent sequences in the target DNA. An external light source excites a donor fluorophore at the 3' end of the

first probe. This then passes its excitation energy on to the second probe, if adjacent, via fluorescence resonance energy transfer (FRET). The 5' end of the second probe is coupled with the acceptor fluorophore which, when activated by FRET, emits measurable light. The intensity of the emission light is proportional to the DNA formed during the ongoing PCR.[163]

4µl of cDNA was added to 2µl of mastermix (LightCycler FastStart DNA master plus, Roche), and made up to 20µl by the addition of 3mM MgCl2, 0.2µM of each 3' and 5' hybridisation probes (TIB MOLBIOL Syntheselabor, Berlin, Germany) and 1µM of each 3' and 5' primers (MWG Biotech). Primers for *BCR–ABL1* and *ABL1* transcript amplification were B2A with NA4 and A2N with NA4 respectively, and the FRET fluorophore probes were BAHP1 and BAHP2 (Table 2.1). The mixture was firstly incubated at 95°C for 10 minutes. Then 40 PCR cycles were carried out, denaturation at 95°C for 10 seconds, annealing at 58°C for 15 seconds and then extension at 72°C for 27 seconds.

The absolute copy numbers of *BCR–ABL1* and *ABL1* transcripts (as an internal control) were calculated by reference to a series of dilution standards ranging from $5x10^5$ to $1x10^2$ copies of linearised plasmids containing a *BCR-ABL1* e13a2 or e14a2 insert. The plasmids were used to generate a standard curve using the LightCycler software. The location of both primers for *ABL1* is within the insert of each of these plasmids, and it was therefore possible to use the same plasmid DNA to generate a standard curve for

ABL1 transcript expression as well. The copy number of *ABL1* transcripts represents both *BCR-ABL1* and wild-type *ABL1* transcripts.

To calculate the *BCR-ABL1/ABL1* ratio, the following formula was used:

$$BCR\text{-}ABL1/ABL1 \text{ ratio} = N_{BCR\text{-}ABL1} / (N_{\text{Total } ABL1} - N_{BCR\text{-}ABL1})$$

where $N_{BCR-ABL1}$ and $N_{\text{total } ABL1}$ were absolute copy numbers of *BCR-ABL1* and *ABL1* transcripts, calculated from the respective standard curves.[112]

2.4.5 Quality of samples.

The quality of a sample is dependent on the sample volume, transportation time and conditions of storage, and is reflected in the control gene *ABL1* transcript number. At our institution the minimum acceptable *ABL1* transcript copy number for clinical reporting is 10,000. Any sample with less than 10,000 copies of *ABL1* is not clinically reported, and therefore was not included in this study.

2.4.6 hOCT1 transporter mRNA analysis

hOCT1 (SLC22A1) mRNA expression levels were determined using diagnostic cDNA on all suitable samples as previously described.[166] This work was carried out by Dr Athina Giannoudis.

2.4.7 Pre-designed TaqMan real time PCR assays

Pre-designed TaqMan® Gene Expression Assays were used in a 384-well assay plate. Each assay consists of a forward and reverse primer at a final concentration of 900nM and a 6-FAM dye-labelled TaqMan probe at a final concentration of 250nM. Table 2.2 lists the TaqMan assays used. This work was kindly performed by Dr Athina Giannoudis.

The concentration of cDNA was determined using the Nanodrop2000 (ThermoScientific, Leicestershire, UK). 100ng of cDNA was used per 20µl reaction consisting of 20x TaqMan gene expression assay and 2x TaqMan gene expression master mix. Each sample was run in triplicate. After loading the reaction mixture, the plate was sealed with an appropriate cover and briefly centrifuged. The real time PCR amplifications were undertaken using an ABI Prism 7900HT System with the following conditions: 50°C for 2 min, 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 secs and annealing/extension at 60°C for 1 min. The relative expression level of a particular gene of a given sample was calculated by the comparative Ct method.[280] The comparative Ct method uses the $2^{-\Delta\Delta Ct}$ formula to achieve results for relative quantification, where $\Delta\Delta Ct$ is the normalised signal level in a sample relative to the normalised signal level in the calibrator sample. A pool of cDNA from 4 normal individuals was used as calibrator and all the samples were normalised to GAPDH as an endogenous control. The RQ Manager software supported by the ABI Prism 7900HT System was used for the analysis.

Table 2.2. Pre-designed TaqMan assays

Assay	ID number
Alox5	Hs01095330_m1
BLT1	Hs019388704_s1
CrkL	Hs00178304_m1
GAPDH	Hs99999905_m1
PP2A (catalytic subunit)	Hs00427259 m1
SET	Hs00853870 g1
PIM1	Hs00270514_m1
SETBP1	Hs00210209 m1

2.4.8 CIP2A mRNA expression

The expression of *CIP2A* was measured using qRT-PCR on a LightCycler 1.5 using LightCycler FastStart DNA MasterPlus SYBR Green I (Roche). *CIP2A* primers and PCR conditions used were as previously described by Come *et al*[93] (forward primer 5′-GAACAGATAAGAAAAGAGTTGAGCATT-3′ and reverse primer 5′-CGACCTTCTAATTGTGCCTTTT-3′). *GAPDH* gene expression in each sample was used as the internal control. PCR conditions were: 50°C for 2 min and a denaturation step at 95°C for 10 min followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 min. The quantification was based on the standard curve method using the BCR-ABL1 positive cell line K562.

2.5.0. CELL CULTURE

2.5.1 Cell lines

Four human BCR-ABL1 positive CML cell lines, KCL22,[281] KY01, LAMA84 and K562[282] were used as a cell line model of CML and a positive control in some experiments, donated by Prof Junia Melo at the LRF Leukaemia Unit, Hammersmith Hospital, London, UK. The human acute monocytic leukaemia cell line U937[283] was used as a BCR-ABL1 negative control. The gastric cancer cell line AGS was used as a CIP2A positive control. Cell lines were cultured under standard tissue culture conditions ($37^{o}C$, 5% $C0_2$ in air, 100% humidity) at approximately $3x10^5$/ml in 'complete culture medium' (RPMI, 10% FCS, 1% L-glutamine and 2% penicillin and streptomycin). Twenty-four hours prior to use the cell lines were resuspended in fresh medium to ensure optimal exponential growth prior to use.

For effects of hOCT1 expression on TKI uptake two stable cell lines were generated, expressing high and low (but not absent) levels of hOCT1 as previously described.[147] This work was carried out by Dr Athina Giannoudis. All control cell lines were cultured and maintained according to instructions supplied by the European Collection of Cell Cultures (ECACC).[272]

2.5.2 Measurement of cell viability

DiOC6/PI (3, 3′-dihexyloxacarbocyanine iodide/ propidium iodide, Sigma-Aldrich) was used to measure cellular viability. DiOC6 is selectively taken up by the charged

mitochondria of living cells but not by the depolarised mitochondria of apoptotic cells. PI stains DNA and is used to differentiate necrotic, apoptotic and normal cells as it cannot permeate the membrane of viable cells. 50µl of cells were gently resuspended in 150µl DiOC6 (40nM in PBS). Following incubation for 15 minutes at 37°C, 200µl of PI (10µg/ml in PBS) was added and the cells were chilled on ice for 30 minutes. Viability was analysed by flow cytometry (FACScalibur), 10,000 events were counted. Live cells are DiOC6 bright / PI dim, early apoptotic cells are DiOC6 dim / PI dim and late apoptotic cells are DiOC6 dim / PI bright.[284]

2.5.3 *In vitro* TKI assay

In some experiments, the effect of tyrosine kinase inhibitors was examined. MNC at a density of 2 x 10^6/ml were cultured either untreated or with 5µM imatinib or 150nM dasatinib or 5µM nilotinib for 24 hours. Imatinib and nilotinib were kind gifts from Novartis (Basel, Switzerland) and dasatinib was a kind gift from Bristol-Myers Squibb (New York, USA).

2.6.0 FLOW CYTOMETRY (FACS)

2.6.1 'In-House' FACS protocol

Cells (~$5x10^5$) were fixed by resuspending in 500µl of 2% paraformaldehyde at pH 7.2 (VWR, Lutterworth, United Kingdom) for 10 minutes at 37°C. Cells were then chilled on ice for 1 minute and centrifuged at 770g for 3 minutes. 500µl of 90% methanol (Fisher Scientific, Leicestershire, United Kingdom) was added to the cell pellet and the cells were vortexed and then incubated on ice for 30 minutes. Cells were then washed (throughout with 1ml incubation buffer containing PBS and 0.5% bovine serum albumin (BSA) (Sigma-Aldrich)), and centrifuged at 770g for 3 minutes. Cells were resuspended in 25µl incubation buffer and left at room temperature for 10 minutes. Appropriate antibody was added (Table 2.3). Cells were vortexed and incubated at room temperature for 40 minutes. Cells were then washed twice and resuspended in 100µl incubation buffer containing 10µg/ml goat anti rabbit/mouse second Alexa Fluor 488 antibody (Invitrogen), incubated at room temperature in the dark for 30 minutes, then washed twice and analysed using flow cytometry (FACScalibur), with Cellquest Pro software (BD) for data analysis.

2.6.2 Caltag FIX & PERM FACS protocol

The Caltag FIX & PERM® (Caltag, Buckingham, UK) reagents firstly fix cells in suspension and then permeabilise them. The nature of the reagents is not disclosed by the manufacturer. This procedure gives antibodies access to intracellular structures but leaves intact the morphological scatter characteristics of the cell. According to the

manufacturer's literature, the 'unique formulation' of FIX & PERM® reagents reduces background staining and allows simultaneous addition of permeabilisation medium and fluorochrome labelled antibodies.

Cells (~$5x10^5$) were fixed using 100µl of reagent A and permeabilised using 25µl of reagent B (Caltag FIX & PERM® reagents). The cells were then washed in PBS containing 0.5% BSA and 0.1% sodium azide (Sigma-Aldrich). Appropriate antibodies were added as given in Table 2.3.

Table 2.3. FACS antibodies

Assay	Control	Primary antibody	Seconday antibody
CIP2A	Mouse IgG2b (BD)	CIP2A (Santa Cruz biotecnology)	Anti mouse Alex fluor 488 (Invitrogen)
CrkL	Normal Rabbit IgG (R&D systems)	CrkL (Santa Cruz biotecnology)	Anti rabbit Alex fluor 488 (Invitrogen)
pCrkL	Normal Rabbit IgG (R&D systems)	pCrkL (Cell Signalling Technology)	Anti rabbit Alex fluor 488 (Invitrogen)
PIM1	Normal Rabbit IgG (R&D systems))	PIM1 (Abcam)	Anti rabbit Alex fluor 488 (Invitrogen)
PP2A	Mouse IgG2a (BD)	PP2A (Millipore)	Anti mouse Alex fluor 488 (Invitrogen)
SET	Normal Rabbit IgG (R&D systems)	I2PP2A (Santa Cruz biotecnology)	Anti rabbit Alex fluor 488 (Invitrogen)
Y^{307}PP2A	Normal Rabbit IgG (R&D systems)	PP2A Y307 (Epitomics)	Anti rabbit Alex fluor 488 (Invitrogen)

R&D systems, Abingdon, UK. Santa Cruz, California, USA. Abcam, Cambridge UK. Millipore, Watford, UK. Epitomics, California, U.S.A.

2.6.3. Triple layer FACS

Cells were fixed, permeabilised and incubated with the appropriate primary antibody as shown in Table 2.3. Cells were then incubated with 5µl of biotinylated anti-rabbit second-layer antibody (Vector Laboratories, Peterborough, UK) for 30 minutes, washed, and incubated at room temperature in the dark for 30 minutes with a third layer antibody, 15µl anti-phycoerythrin streptavidin (SA PE) antibody (BD). Cells were washed twice and analysed with 10,000 events being collected.

2.6.4 Measurement of LTB4 receptor BLT1

5ml of peripheral blood was collected into EDTA. Erythrocytes were depleted using red cell lysis buffer as described in section 2.3.1. Total leukocytes were then incubated with Leukotriene B4 Receptor 1 (BLT1/LTB4R1) (R&D systems) and anti mouse IgG1 antibody (BD) as control antibody, in the dark for 30 minutes with shaking. Cells were then washed in PBS containing 0.5% BSA and analysed by FACS.

2.6.5 BCR-ABL1 protein detection flow cytometry protocol

Using the BCR-ABL1 protein detection kit (kind gift from BD) the manufacturer's protocol was followed. $1x10^7$ cells were washed in PBS containing 5% FCS, pelleted, and resuspended in 250µl of pre-treatment reagent and incubated on ice for 10 minutes. Cells were centrifuged at 500g for 5 minutes at 4°C, washed in PBS and centrifuged again. The supernatant was discarded and 100µl of BD lysing solution was added to the

cell pellet and incubated on ice for 15 minutes, followed by centrifugation at 20,000g for 10 minutes at 4°C. 50µl of supernatant was incubated with 50µl of capture beads and 50µl detector reagent in the dark for 2 hours, then washed with 1ml BD CBA wash buffer and centrifuged at 770g for 5 minutes. Beads were resuspended in 300µl BD CBA wash buffer and data acquired and analysed using a FACSCanto II.

The reported BCR-ABL1 MFI (mean fluorescence intensity) for a sample was determined as the MFI for the sample, minus the MFI value of the negative control plus two standard deviations.

The BCR-ABL1 protein FACS assay was performed in collaboration with Miss Jemma Fagan.

2.7.0 WESTERN BLOTTING

2.7.1 Protein Determination

200µl of 1% SDS lysis buffer containing 1% SDS, 50mM Tris, 5mM EDTA, 10% glycerol, chymostatin, leupeptin, aprotinin, pepstatin A and antipain 1mg/ml (Sigma-Aldrich) was added to the cell pellet and sonicated. The lysates were then heated at 95°C for 10 minutes then placed on ice to cool. Protein determination was performed using the BioRad DC protein assay (BioRad, Hertfordshire, UK). Protein standards of 0-3µg were made using BSA diluted in 1% SDS lysis buffer (1% SDS, 50mM Tris, 5mM EDTA, 10% glycerol). 5µl of protein standards and samples were added into a 96 well flat bottom plate (Fisher Scientific), using a positive displacement pipette and tips. 20µl of reagent S was added to 1ml of reagent A. 25µl of reagent A/S mixture was added to each well followed by 200µl of reagent B (Reagents A, B and S were provided in the BioRad DC protein assay kit). The plate was incubated at room temperature for 15 minutes and then the plate was read using a spectrophotometer at 750nm.

2.7.2 Western blotting

Lysates were prepared using SDS lysis buffer and boiled for 10 minutes. Samples were loaded by either cell number or protein concentration (20µg) and electrophoresed in 15% polyacryamide gels in Tris/glycine running buffer[285] using Bio-Rad protean II xi cell electrophoresis system. Following electrophoresis, proteins were transferred for Western blotting to Immobilon-P membranes (Millipore). Membranes were blocked with 5% w/v skimmed milk (Tesco) or 5% BSA (Sigma-Aldrich) in Tris buffered saline (TBS- T)

(10mM Tris pH 7.4, 150mM NaCl, and 0.1% Tween-20) and incubated with primary antibody overnight at 4°C with shaking. Membranes were then washed thoroughly with TBS-T and incubated with secondary antibody for 30 minutes at room temperature. Membranes were again washed thoroughly and blots were developed using ECL (enhanced chemiluminescence) (Millipore). Digital images were obtained using the LAS-1000 imaging system and analysed using Aida Image Analyser software (Raytest, Straubenhardt, Germany).

2.7.3 Immunoprecipitation

$1x10^7$ cells were lysed in 500µl RIPA buffer (Tris 50mM, NaCl 150mM, SDS 0.1 %, Na deoxycholate 0.5 %, NP40 1%, sodium vanadate 1mg/ml and PMSF (phenylmethylsulfonyl fluoride 1mg/ml)), incubated on ice for 20 minutes, and then centrifuged at 12,000g for 30 minutes at 4°C. The supernatant was transferred into a new 1.5ml Eppendorf tube. 20µl of protein A and G beads (Sigma-Aldrich) were washed twice in RIPA buffer and then added to the cell lysate, and agitated for 1 hour at 4°C. The lysates were centrifuged at 12,000g for 2 minutes and then transferred into a new 1.5ml Eppendorf tube. The appropriate antibody was added to the cell lysate (1µg per 10^7 cells) and incubated for 2 hours at 4°C (rotating). 20µl of protein A and G beads were washed twice in RIPA buffer and then added to the cell lysate and incubated overnight at 4°C (rotating). Beads and antibody alone were used as a control. Samples were pelleted, washed in TBS-T and resuspended in 20µl of Double strength sample (DSSB) buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol (Sigma-Aldrich), 0.004% bromophenol blue

(Sigma-Aldrich) and 0.125M Tris HCL (Sigma-Aldrich)). Western blotting was performed as described above.

2.8.0 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

ELISA assays protocols were developed for the detection of tyrosine kinase activity, c-Myc total protein and pS^{62}c-Myc. For the detection of LTB4 a commercially available assay was used.

2.8.1 pTyr and c-Myc ELISA

Whole-cell lysates were prepared in NP-40 lysis buffer (1% NP40, 150 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, and 1% protease inhibitor, pH 8.0, Sigma-Aldrich). Lysates were diluted in 50mM carbonate-bicarbonate coating buffer (Sigma-Aldrich), added to each well of an Immulon 4 HB 96-well flat-bottom ELISA plate (Fisher Scientific) in triplicate (5µg protein in 100µl carbonate-bicarbonate buffer/well) and plates were then incubated overnight at room temperature with shaking. Following incubation, plates were washed with TBS-T four times; surfaces were blocked with 100µl 5% BSA in TBS-T (except those wells reserved for chromagen blanks) and plates incubated for 1 hour at room temperature with shaking. Plates were then washed as previously. Plates were incubated with 100µl of TBS-T containing primary antibody and 5% BSA, for 2 hours at room temperature on a shaker. Plates were washed with TBS-T four times and incubated with 100µl per well of 1:1000 anti-mouse or rabbit IgG–horseradish peroxidase secondary antibody (New England Biolabs, Hitchin, UK) for 1 hour at room temperature

on a shaker. Plates were then washed four times with TBS-T, and then 100µl chromagen substrate (tetramethylbenzidine, Sigma-Aldrich) was added to each well and plates incubated for 20 minutes in the dark. The reaction was stopped by adding 50µl 2M HCl and plates were then read at 460 nm absorbance. Negative control wells were included in the assay (lysate only and antibody only) and these values were subtracted as background from each relevant test well.

For tyrosine kinase activity the primary antibody was 4G10 mouse anti-phosphotyrosine (P-Tyr) antibody (Upstate Biotechnology, New York, USA).

For assaying c-Myc and pS^{62}c-Myc status, Myc antibody (New England Biolabs) and pS^{62}c-Myc antibody[92] (Abcam) were used respectively.

2.8.2.Leukotriene B4 ELISA

LTB4 standards (3000, 750, 188, 46.9 and 11.7pg/ml) were prepared and the assay was performed according to the manufacturer's protocol (Cambridge Biosciences, Cambridge, UK). 100µl of the specific standard, sample or assay buffer was added in duplicate to appropriate wells of a 96-well plate and a further 50µl of assay buffer to the negative control wells. 50µl of LTB4 conjugate and 50µl of LTB4 antibody, supplied in the kit, were added to the appropriate wells. The plate was incubated at room temperature, with shaking, for 2 hours. Following incubation the plate was washed three times with wash

solution (provided in the kit). 5µl of conjugate was added to the total activity wells, followed by 200µl of pNpp substrate solution to every well. The plate was incubated again, at 37°C with shaking, for a further 2 hours. 50µl of stop solution (2M HCl) was then added to all wells. The absorbance was immediately read at 405nm using a BioTeK µQuant plate reader. The mean optical density of the negative control wells was subtracted from the optical density reading of all wells, to eliminate "background noise" of each well.

2.9.0 PP2A ACTIVITY ASSAY

PP2A phosphatase assays were carried out using the PP2A IP phosphatase assay kit (Millipore). The manufacturers protocol for this assay was optimised by Professor Danilo Perrotti's laboratory. Cells were pelleted and washed in 1ml of 10mM HEPES buffer and then resuspended in 100µl of hypertonic buffer (10mM HEPES, 1% NP-40, 0.5 M NaCl, 10% Glycerol, 1mM PMSF, 5mM Benzamidine and protease inhibitors cocktail) and left on ice. Cell lysates were subjected to four freeze thaw cycles, consisting of 30 seconds in ethanol and dry ice and then heated to 37°C. Lysates were then sonicated and kept on ice for 20 minutes, followed by centrifugation at 14,000g for 20 minutes at 4°C. Protein concentration was determined as described in section 2.7.1. 25µl protein A beads were washed in pNPP ser/thr assay buffer (50mM Tris-HCl, pH 7.0 and 100mM $CaCl_2$) and resuspended in 400µl pNPP Ser/Thr assay buffer. 5µg anti-PP2A, c subunit, clone 1D6 (as provided in the kit) and 100µg of cell lysate were added to the beads, and then samples were agitated for 2 hours at 4°C. Samples were centrifuged at 14,000g for 3 minutes at 4°C and the supernatant was discarded. Beads were washed three times in TBS

and once in 1ml of pNPP Ser/Thr assay buffer and resuspended in 25µl pNPP Ser/Thr assay buffer. 60µl of phosphopeptide was added to the washed immunoprecipitated beads. Lysates were agitated for 10 minutes at 30 ºC, then centrifuged at 14,000g for 3 minutes and the supernatant was transferred to a new 1.5ml Eppendorf tube. 25µl of the supernatant was added to a 96 well plate in triplicate. pNPP ser/thr assay buffer was used as an assay negative control. Phosphate standards provided with the kit were also added to the plate. The Malachite phosphate detection solution was prepared by adding 1000µl of solution A to 10µl of solution B and 300µl Malachite phosphate detection solution was added to every well. The plate was incubated at room temperature for 10 minutes and plates were then read at 650nm absorbance. The standard curve was used to determine the pico moles of phosphates released from phosphopeptide by PP2A activity (absorbance depends on PP2A activity of the samples).

2.10.0 CIP2A SMALL INTERFERING RNA (siRNA) TREATMENT

K562 and LAMA84 cells were washed three times in PBS. $1x10^6$ cells were resuspended in 100µl solution V Nucleofector Kit V (Lonza, Verviers, Belgium) and 100nM siRNA was added to solution V (CIP2A siRNA and control siRNA, Santa Cruz) or CIP2A siRNA (Integrated DNA technologies, Leuven, Belgium). Samples were transferred to an Amaxa vial, and nucleofected using the Amaxa instrument pre-set programme T16. After nucleofection the cells were immediately transferred into pre-warmed culture medium, and cultured for 72 hours in a 24 well plate prior to analysis.

2.11.0 STATISTICAL ANALYSIS

Statistical analysis and comparisons were performed using the statistical programme SPSS 16.0 (SPSS Inc. Chicago, USA). Fishers and Mann-Whitney tests were used to test if the data were significant; details are given where appropriate with each experiment. SPSS was also used to draw box and whisker plots as well as Kaplan-Meier plots.

CHAPTER THREE - A population study of imatinib in chronic myeloid leukaemia

3.1.0. INTRODUCTION

Imatinib has undoubtedly saved and improved the quality of lives of many thousands of patients with CML and it has become the treatment of choice for newly diagnosed chronic phase patients. A recent report suggests that modern drug treatment may produce superior outcomes compared to allogeneic stem cell transplantation (SCT).[120] However, SCT remains the only proven curative therapy, but is only an option for about 25% of patients and carries substantial risks.[20]

At the time of conducting this study the only data available on the efficacy of imatinib for newly diagnosed CML patients were from a single trial, IRIS, which was carried out under the supervision of the imatinib manufacturer Novartis. The development of imatinib and the IRIS clinical trial results have been discussed in the general introduction section 1.5.5. Briefly the IRIS trial reported a five year cumulative CCR rate of 87% and an estimated OS of 89%. However, 30% of patients were excluded from the five year assessment for various reasons. EFS and disease progression rates were therefore only calculated on those patients still continuing imatinib treatment at five years and not those who had switched treatment because of treatment failure or for other reasons.

As for any clinical trial, patients who enrolled in the IRIS clinical trial differ from the general population - for example patients over the age of 70 were excluded, together with those with conditions such as diabetes mellitus, angina, or a history of another

malignancy within the past five years. However, such patients are seen in CML clinics on a regular basis. It was therefore important to establish if the IRIS trial outcome data could be reproduced in a more general, unselected CML population. The work contained within this chapter is the outcome of the analysis undertaken to assess the clinical outcome of all newly diagnosed CML CP patients receiving imatinib 400mg daily in our healthcare area.

3.2.0. PATIENT COHORT

All patients aged 16 or over with CML newly diagnosed between January 1st 2003 and June 30th 2006 were included in this study. Patients diagnosed before this period were excluded since the timing of imatinib approval by primary care trusts in 2002 varied across our geographical area. Younger patients were excluded as imatinib usage in children is not universal, and molecular monitoring in paediatric patients was not performed routinely at that time. Patients were included in the assessment of imatinib efficacy if they received imatinib 400mg daily from original diagnosis (preceded only by up to six weeks of hydroxycarbamide).

Considerable effort was made to include all CML cases, firstly from the records at the molecular diagnostic centre, secondly by contact with local haematologists and thirdly by cross-checking against Philadelphia translocation positive (Ph^+) results at the Liverpool, Manchester and Cardiff regional cytogenetics centres which cover our geographical area.

For each case, local haematologists verified that each case had never received prior interferon-α or alternative chemotherapy. Patient characteristics are shown in Table 3.1.

Table 3.1. Summary of patients' characteristics – population study

		Total
Number of patients		88
Mean age, (range years)		53 (18-87)
Sex (M/F)		41/47
Sokal score	High	28
	Intermediate	29
	Low	20
	No data	11

3.3.0. RESULTS

3.3.1. Defining the clinical cohort.

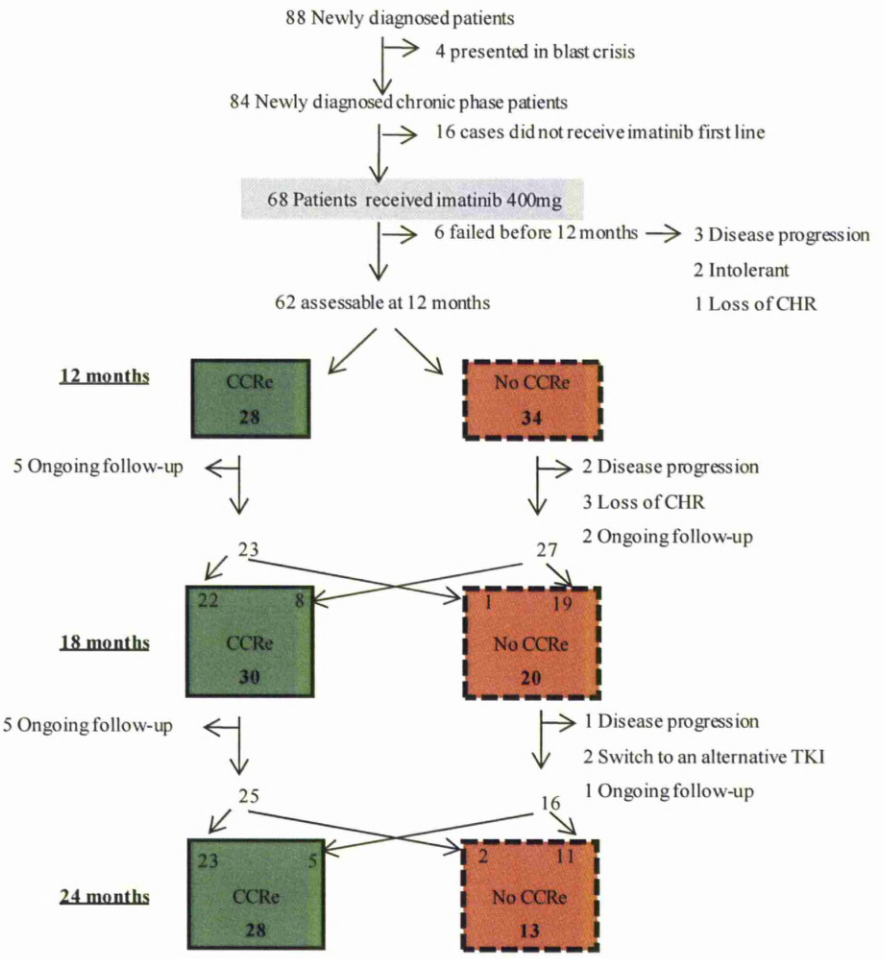
Over the 42 months of the study, 88 new cases of CML were seen, giving an annual incidence of 1.2 cases per 100,000 population per annum. These comprised 41 men and 47 women, and their median age at diagnosis was 53.4 years (range 18.6 – 86.8 years). Of these, four presented in blast crisis and were treated with chemotherapy prior to imatinib and then stem cell transplant (SCT), and therefore were excluded from further study. Of the 84 presenting in chronic phase, 16 cases were not assessable for the effect of imatinib as they first received interferon-α (seven cases) or an elective SCT (one case), or because insufficient follow-up data were available (eight cases). Analysis was therefore carried out on all 68 assessable cases that presented in chronic phase and received imatinib from initial diagnosis.

3.3.2. Response rates to imatinib treatment (400mg)

A flow diagram of patient outcome is shown in Figure 3.1. During the first 12 months of treatment, three patients progressed to blast crisis, of whom two died and one achieved a second chronic phase during which they underwent a SCT. Two patients had serious side effects attributable to imatinib treatment, one Stevens-Johnson syndrome – a serious exfoliative skin disease affecting the skin and mucous membranes that is usually the result a drug reaction,[286] and one case of hepatotoxicity, both of whom were successfully treated with an alternative TKI. One further patient lost an initial CHR, and proceeded to

SCT. Six cases therefore failed imatinib before 12 months, leaving 62 cases assessable at 12 months.

Figure 3.1. A flow diagram of patients achieving CCRe.



3.3.3. Imatinib response in patients assessable at 12 months.

Of the 62 cases that were assessable for response at 12 months, 28 (41%) had achieved a complete cytogenetic response (CCRe). Twenty-three of these 28 cytogenetic responders were assessable at 18 months and 22 remained in CCRe; the remaining case developed central nervous system (CNS) blast crisis. Of the 34 cases who had not achieved CCRe at 12 months, eight patients went on to achieve a CCRe at 18 months. Twelve of the original 68 imatinib treated patients were not assessable at 18 months (three cases lost their CHR and proceeded to SCT, two died of disease progression and seven cases had not reached the 18 month assessment point).

By 24 months, a total of six (9%) of the original 68 imatinib treated patients had progressed to advanced phase, and 10 further cases remained in chronic phase but had received SCT or switched to an alternative TKI because of intolerance (three cases) or failure to achieve/maintain CCRe. The overall CCRe rates were 41% (28 of 68 patients) at 12 months, 49% (30 of 61 assessable cases) at 18 months, and 51% (28 of 55 assessable cases) at 24 months (Table 3.2).

Table 3.2. CCRe response rate.
Panel A: All 88 patients and **B:** the 68 patients who received imatinib from initial diagnosis.

A.

All patients				
	Total	12 months	18 Months	24 months
Number of cases assessable	88	83	81	80
Low Sokal score	20	55% (11/20)	53% (10/53)	42% (8/19)
Intermediate Sokal score	29	24% (6/25)	36% (9/25)	36% (9/25)
High Sokal score	28	48% (13/27)	41% (11/27)	41% (11/27)
No data	11	27% (3/11)	40% (4/10)	44% (4/9)
Overall CCRe	88	40% (33/83)	42% (34/81)	40% (32/80)

B.

Patients receiving imatinib from initial diagnosis				
	Total	12 months	18 Months	24 months
Number of cases assessable	68	68	61	55
Low Sokal score	18	50% (9/18)	52% (9/17)	43% (7/16)
Intermediate Sokal score	22	27% (6/22)	38% (6/21)	42% (8/19)
High Sokal score	23	52% (12/23)	61% (11/18)	68% (11/16)
No data	5	20% (1/5)	40% (2/5)	50% (2/4)
Overall CCRe	68	41% (28/68)	49% (30/61)	51% (28/55)

CCRe was used as a conservative and confirmed predictor of treatment success or failure at 12, 18 and 24 months.[111,160] At 24 months, 49% (27 of 55 assessable cases) had failed imatinib because of either progression to blast crisis (six cases, all fatal), failure to achieve/maintain CCRe (19 cases, of which four lost CHR as well) or intolerance (two

cases). Fourteen of the 15 failures who remain in CHR and without disease progression have achieved and remain in CCRe at latest follow-up, following either SCT or an alternative tyrosine kinase inhibitor (TKI).

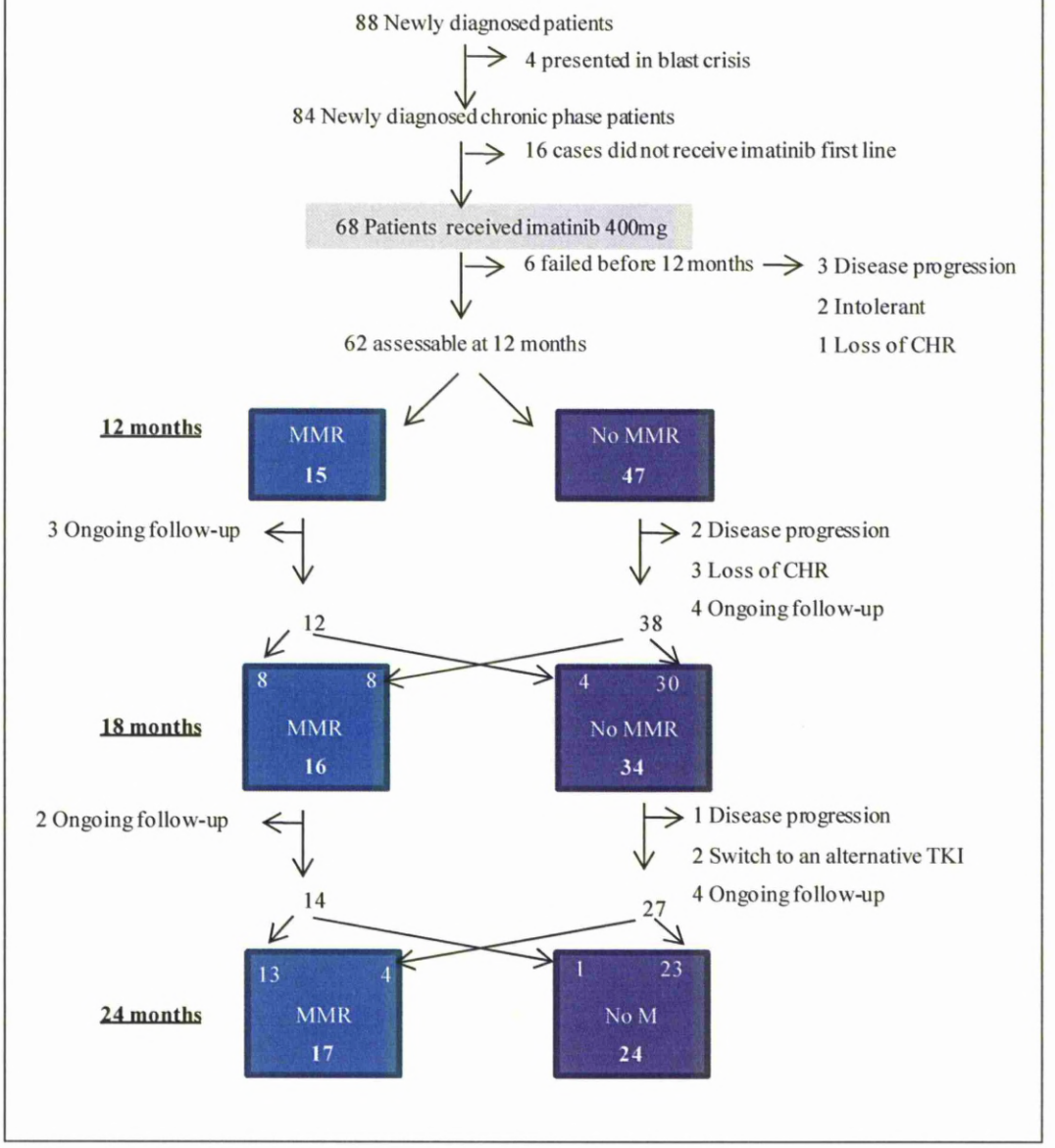
The CCRe rates were stratified by the patients' Sokal score. The purpose of this was to demonstrate that there was an even distribution of patients between each Sokal risk group. Patients were stratified as follows: 26% low, 32% intermediate and 34% high risk; thus patients were considered to be evenly distributed. The lower CCRe rates observed cannot therefore be attributed to a bias of high Sokal scoring patients within this study. A similar Sokal score range was observed in a single centre study from the Hammersmith Hospital, London.[287] The distribution of patients within the IRIS study was 52.5% low, 29% intermediate and 18.5% high risk.[17] Given that the IRIS study censored patients when they discontinued treatment it is plausible that the 'missing' patients from IRIS were predominately high risk Sokal score patients. Superior IRIS response rates compared to the present study might therefore be attributed to the bias in the patient cohort for low risk Sokal score patients.

The IRIS study reported 12 month CCR rates of 79% low, 67% intermediate and 49% high risk. At 18 months the CCR rate was reported for the high risk group as 56.3%, which is comparable with 61% at 18 months in this population study. While the IRIS study demonstrated that a patient's Sokal score correlated with achievement of CCR, in the present population based study this was not the case (Table 3.2). Patients with high

risk Sokal scores had a higher rate of CCRe at all time points compared to low and intermediate risk groups. It is possible that the number of patients in this study is too low for an association between CCR and Sokal score to be observed. Alternatively the follow up period may not be long enough.

In the present study, MMR rates were 22% at 12 months, 26% at 18 months and 31% at 24 months. Five patients (8%) lost MMR during the study. Three patients had a transient loss of MMR but regained MMR at a later time point while still on imatinib treatment. One patient developed the T315I BCR-ABL1 kinase domain mutation and subsequently died. The remaining patient lost both CCRe and MMR and switched to nilotinib treatment. At latest follow up she remains in CMR (Figure 3.2).

Figure 3.2. A flow diagram of patients achieving MMR.

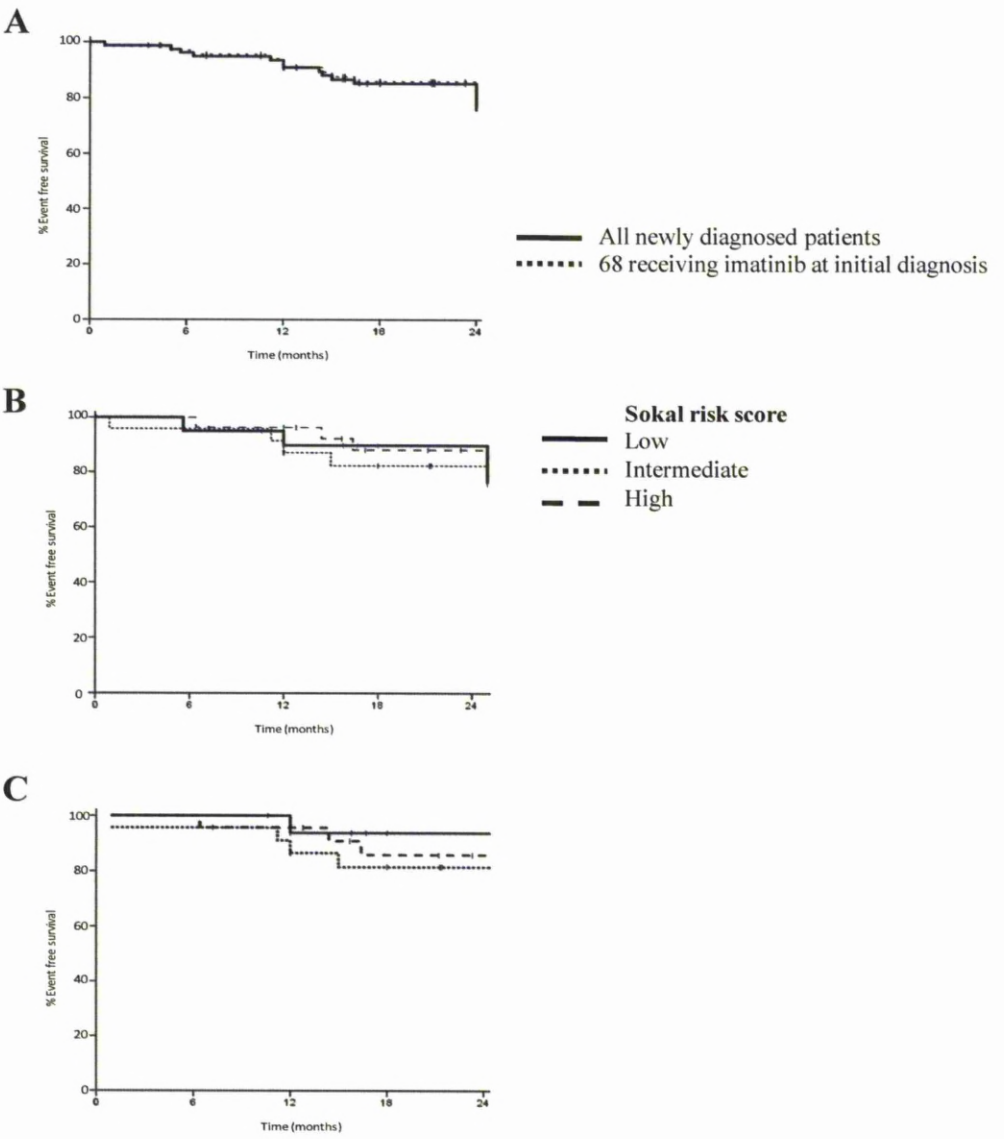


3.3.4. Event free survival is not associated with Sokal score in imatinib treated patients.

In the IRIS study and the present study, event free survival (EFS) was defined as death from any cause, progression to blast crisis or accelerated phase, loss of haematological response or loss of cytogenetic response.[17] Patients who presented in blast crisis are not eligible for a disease progression event since they presented in blast crisis.

Figure 3.3 shows that in the present study, the EFS for all patients was 95% at 12 months and 80% at 24 months, with no difference in EFS between all 88 patients and the 68 entered into the study (Panel A). EFS was also stratified by Sokal score, and no statistically significant difference was observed between the Sokal score risk group and EFS for all 88 patients (Figure 3.3B) or the 68 who entered the study (Figure 3.3C). These data suggest that Sokal score may not be an appropriate calculation of disease risk for imatinib treated patients given that it was developed in the pre-imatinib era.[155] A recent study performed by the European LeukaemiaNet on 2060 newly diagnosed CML patients treated with imatinib at initial diagnosis reported that the Sokal score discrimination was only statistically significant for high risk patients. The authors developed a new scoring calculation known as the 'EUTOS score' which they suggest is more accurate at stratifying imatinib treated patients into risk groups.[160] Unfortunately the parameters required to perform the new calculation were not available for this cohort of patients therefore it could not be tested.

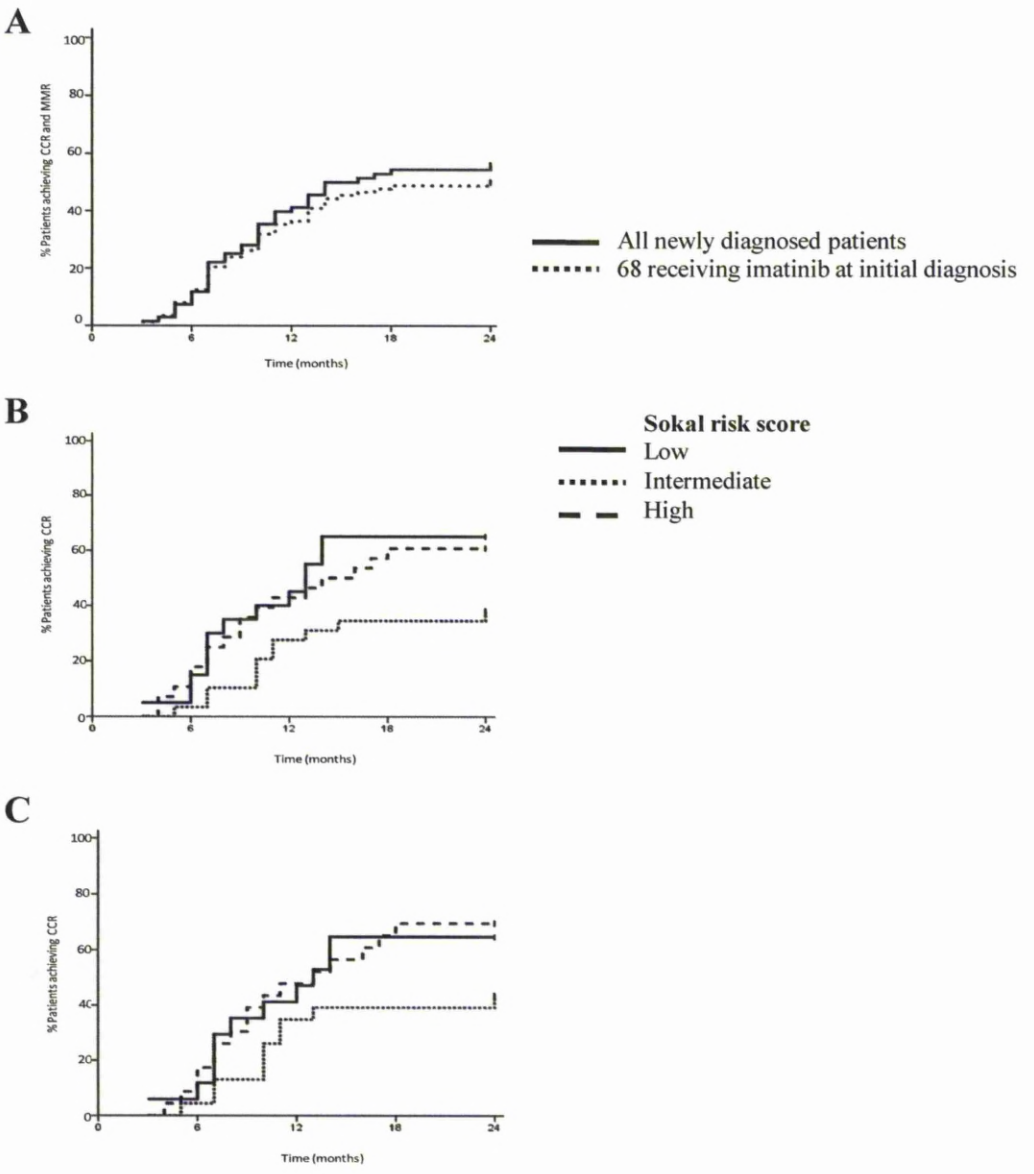
Figure 3.3. Kaplan-Meier estimates of event free survival.
Panel A: Comparison between all 88 patients and the 68 patients who received imatinib from initial diagnosis. **Panel B:** Outcome according to Sokal score for all 88 patients. **Panel C:** The 68 imatinib treated patients. No statistically significant differences were observed for either group (SPSS statistical package – Mantel-Cox log rank test).



3.3.5. Time to achieve CCRe is not associated with Sokal score in imatinib treated patients.

No statistically significant difference in time to achieve a CCRe was observed between all 88 patients and the 68 patients whom entered the study (Figure 3.4A). However, this Kaplan-Meier graph is a cumulative estimate of time to achieve CCRe and does not take into account loss of response. When the patient cohort was stratified by Sokal score no statistically significant difference in time to response was observed (Figure 3.4B and C). These data further confirm the idea that Sokal score cannot predict achievement of CCRe or EFS in imatinib treated patients.

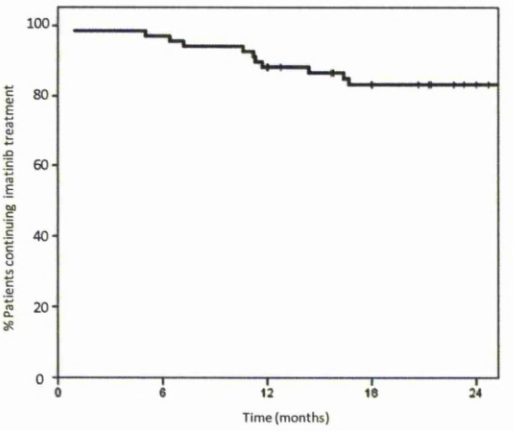
Figure 3.4. Kaplan-Meier estimates of time to achieve CCRe.
Panel A: Comparison between all 88 patients and the 68 patients who received imatinib from initial diagnosis. **Panel B:** Outcome according to Sokal score for all 88 patients. **Panel C:** The 68 imatinib treated patients. No statistically significant differences were observed for either group (SPSS statistical package – Mantel-Cox log rank test).



3.3.6. Continuation of imatinib treatment.

After two years follow-up 80% of patients who received imatinib at initial diagnosis continue to receive imatinib 400mg daily as treatment for CML. Failure to respond to treatment in this study is defined as not achieving CCRe.[160] Although 49% of patients had failed imatinib therapy at 24 months (Figure 3.5), the fact that a high percentage of patients remain on imatinib treatment reflects the lack of alternative treatment options for chronic phase CML patients at the time of study, which was limited to SCT or imatinib since second generation TKIs were not available.

Figure 3.5. Patients continuing imatinib treatment.
Kaplan Meier curve showing the percentage of patients continuing to receive imatinib 400mg daily.



3.4.0. DISCUSSION

At the time of conducting this study there were several hundred publications on various clinical aspects of imatinib, but only three independent series describing clinical outcome in previously untreated chronic phase CML patients; the IRIS trial, and studies from the MD Anderson Hospital, Texas, USA and the Hammersmith Hospital in London.[18,287,288] In a single centre study of 279 newly diagnosed patients presenting to the MD Anderson Hospital 87% achieved a CCR, with an estimated three year survival rate of 96%.[288] A single centre study conducted at Hammersmith Hospital evaluated the response to imatinib first-line in 204 newly diagnosed CML patients.[287] The five year cumulative CCR and MMR rates were 82.5 and 50.1% respectively. Patients who achieved a CCR by 12 months had a significantly better PFS and OS compared to those patients who did not, and achievement of MMR had no additional benefit in this cohort, which contrasts with the reported data by the IRIS investigators.[164] By five years 25% of patients had discontinued imatinib and cumulative incidence of loss of CCR at 48 months was 17%. The study concluded that a third of patients need better treatment. The above studies provide insight into the efficacy of imatinib in CP CML patients, though none of these three studies were population based.

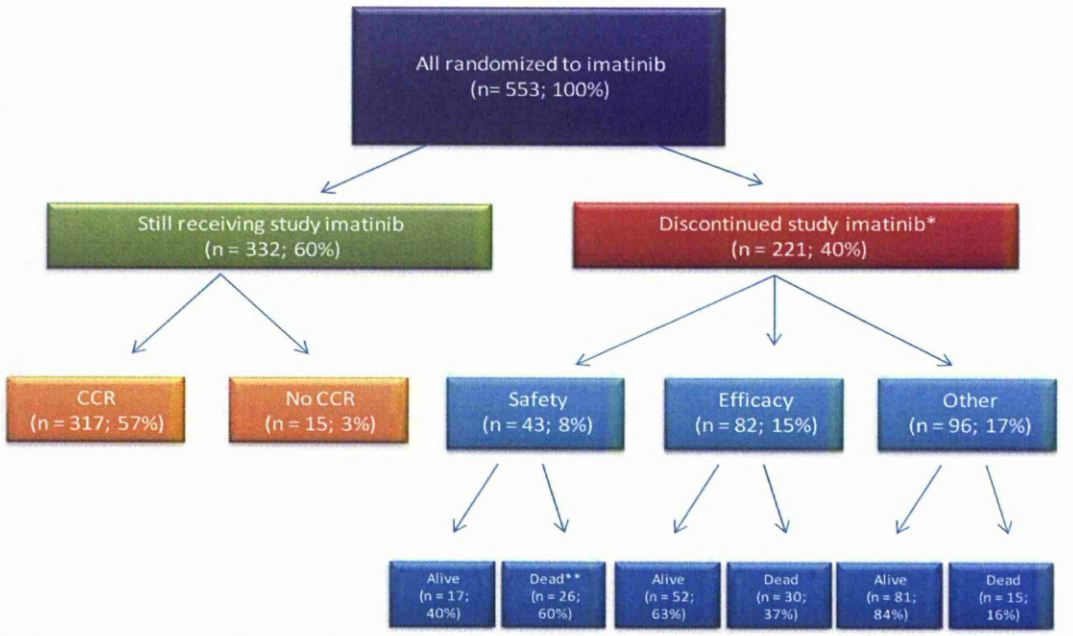
The present data indicate that within our geographical area, following 24 months of imatinib treatment, the CCRe rate is only 51%, meaning that nearly half of all patients will fail treatment at two years. This differs from the response rates seen within the IRIS study, which demonstrated that after five years of imatinib therapy, 83% of patients are alive and remain in chronic phase.[18] The cumulative rates of CCR among the patients receiving 400mg of imatinib were reported as 69%, 76%, and 87% at 12, 18 and 60 months respectively.[18] In this population study evaluation of the patients' Sokal score revealed an even distribution

between the risk groups' ruling out the hypothesis that there were more high risk patients presenting within the North-West explaining our lower rates of CCRe and MMR.

Exclusion criteria were applicable for inclusion within the IRIS trial including: an age limit (≥ 18 and ≤ 70 years of age), patients who have received other investigational agents, patients who received prior chemotherapy and patients with uncontrolled medical disease such as diabetes mellitus, thyroid dysfunction, neuropsychiatric disorders, infection or angina. In addition to the exclusion criteria there was a geographical bias for patients entering the IRIS study, since only large specialist centres offered the IRIS trial. Patients being treated at smaller district general hospitals were often not entered due to the time and cost implications of travelling frequently to the larger hospital.

A further major issue within the IRIS data is the censoring of 'failed' patients. Unlike the present analysis which includes all patients in the final analysis, the IRIS trial censored patients who had failed to stay on imatinib treatment, at the point of imatinib discontinuation. Thus as time evolves, the IRIS dataset is increasingly selected for patients who have done well.

Figure 3.6. IRIS 7 year analysis.



*Patients may have continued imatinib off study.
**Including primary discontinuation

At the American Society of Hematology (ASH) annual meeting in 2008 the IRIS data were re-analysed to include these previously excluded patients (Figure 3.6). After seven years of follow up over 40% of patients initially entered into the trial had been excluded from analysis.[289] Of the 40% of patients who discontinued treatment 8% were due to safety, 15% due to lack of efficacy which included progression to AP or BC, loss of response, and unsatisfactory therapeutic effect, and 17% other reasons including SCT, withdrawn consent and lost to follow-up.

The findings of this present population study were recently substantiated by an abstract presented at the European Haematology Association (EHA) annual meeting 2010.[290] A population study examining a more recent cohort of patients in the West of Scotland and

Lothian reported that only 46.2% of patients initially treated with imatinib remain on treatment (median time to discontinuation of imatinib treatment 13.2 months). These data together with the present findings indicate that caution is needed in extrapolating clinical trial data to the general CML population.

With increasing clinical experience, it is becoming apparent that some patients can become resistant to imatinib.[178,291] Imatinib resistance is multifactoral, including BCR-ABL1 kinase domain mutations, BCR-ABL1 gene amplification, or changes in drug transporter expression such as hOCT1 and ABCB1[33,291,292] Second generation TKIs dasatinib and nilotinib are now licensed for first-line therapy based on data from ongoing phase III clinical trials (DASISION and ENESTnd). These trials suggest that both dasatinib and nilotinib are superior drugs when compared with standard dose imatinib,[146] although whether these superior response rates are maintained in years two and beyond remains unknown.[146,152] It will be interesting to see if the clinical trial findings from these second generation TKIs can be replicated in the more general CML population. A population study of second generation TKI treatment within our area is at present being conducted.

Currently it is impossible to prospectively identify patients who will respond to imatinib treatment at diagnosis. Clinical decisions would be greatly facilitated if it was possible to accurately predict patients who are unlikely to achieve cytogenetic and/or molecular response prior to commencing treatment, and this would facilitate the individualisation of therapy. Therefore the aim of this thesis is to try and identify biomarkers which may predict eventual clinical outcome in newly diagnosed CML patients.

CHAPTER FOUR - BCR-ABL1 tyrosine kinase activity as determined by the pCrkL/CrkL ratio at diagnosis is predictive of treatment outcome in chronic myeloid leukaemia.

4.1.0 INTRODUCTION

Prognostic scoring methods based on simple haematological parameters that were devised in the pre-imatinib era (i.e. Sokal score[155]) may still distinguish cohorts of patients with different outcomes on imatinib treatment.[18] Similarly the level and activity of the imatinib uptake transporter hOCT1 may also correlate with clinical outcome.[166,293] However, none of these parameters are sufficiently powerful to prospectively predict patients destined to fare poorly on imatinib treatment. Clinical decisions would be greatly facilitated if it were possible, prior to commencing imatinib treatment, to accurately predict patients who are unlikely to achieve a cytogenetic response, as these patients could then be offered alternative treatments such as SCT or a second generation TKI.

A rational approach to treatment optimisation would be to use an assay which measures the degree of BCR-ABL1 tyrosine kinase activity and then how imatinib modifies this either *in vivo* or *in vitro*. BCR-ABL1 tyrosine kinase activity has previously been assessed by Western blotting.[197,198] However it is known that CML MNC release a proteolytic degradative activity upon lysis which rapidly and selectively degrades the BCR-ABL1 protein leading to an under estimation of its activity.[189,199,294] Thus, direct assessment of BCR-ABL1 tyrosine kinase activity in CML primary cells is experimentally problematic. As discussed in the general introduction (section 1.6.8), White *et al*[197] investigated imatinib induced *in vitro* inhibition of the pCrkL and CrkL levels by Western blotting and produced a ratio. The authors reported that the imatinib IC50 (defined as the imatinib concentration that reduces the pCrkL/CrkL ratio by 50%) was predictive of subsequent clinical outcome. Western blotting can be time consuming and also requires a large number of cells ($>10^6$) which may be difficult to obtain from CML patient samples especially once treatment has begun. A pCrkL FACS method was

subsequently developed by Hamilton *et al.*[199] Flow cytometry results were comparable to those produced by Western blotting. Flow cytometry has the advantage that it requires fewer patient cells (~ 10^3 cells), produces quantitative values and results can be obtained rapidly. However, unphosphorylated CrkL was not assessed, so it was not possible to calculate a pCrkL/CrkL ratio as suggested by White *et al.*[197]

The aim of this chapter was to optimise and further develop techniques for the detection of both pCrkL and CrkL. At the time of commencing this work pCrkL and CrkL could only be detected by Western blotting,[197,199] and during the project the detection of pCrkL was reported by flow cytometry.[199] The main focus of this chapter was to investigate the predictive value of pCrkL and CrkL in newly diagnosed CP CML patients.

4.2.0. RESULTS

4.2.1. Detection of pCrkL and CrkL by Flow cytometry

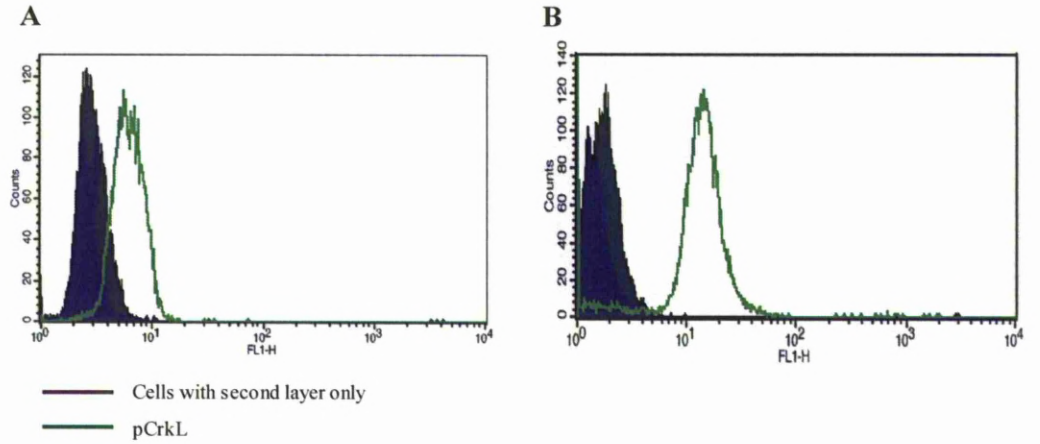
The initial aim of this work was to develop a FACS assay for the detection of pCrkL and CrkL which would be clinically more favourable and easier to use as a diagnostic tool than Western blotting. A considerable amount of time was spent optimising the FACS assay. The following parameters were investigated as likely to affect the final result:

- Cellular fixation and permeabilisation - comparison of 'Caltag fix and perm kit' and the 'in house' protocol of paraformaldehyde and methanol fixation.
- Saturating antibody concentration.
- Signal detection – double or triple layer signal amplification.
- Nature of second layer fluorophores.
- Source of control antibody /non-specific binding.
- Reproducibility (sensitivity).
- Cell source – fresh or frozen.

4.2.1.1. Fixation and permeabilisation

Various methods have been published for the fixation and permeabilisation of cells for FACS analysis. Hamilton *et al*[199] used a commercially available kit from Caltag to fix and permeabilise cells in order to assess the CrkL phosphorylation status in CD34+ cells. This section addresses whether the findings of Hamilton *et al*[199] are reproducible in our laboratory, and whether our 'in-house' fixation and permeabilisation protocol (using paraformaldehyde and methanol) offers any further improvement.

Figure 4.1. Detection of pCrkL in K562 cells by FACS using different methods of fixation and permeabilisation. (Representative plots are shown).
Panel A: Caltag FIX & PERM® kit. **Panel B:** 2% paraformaldehyde to fix and 90% methanol to permeabilise.

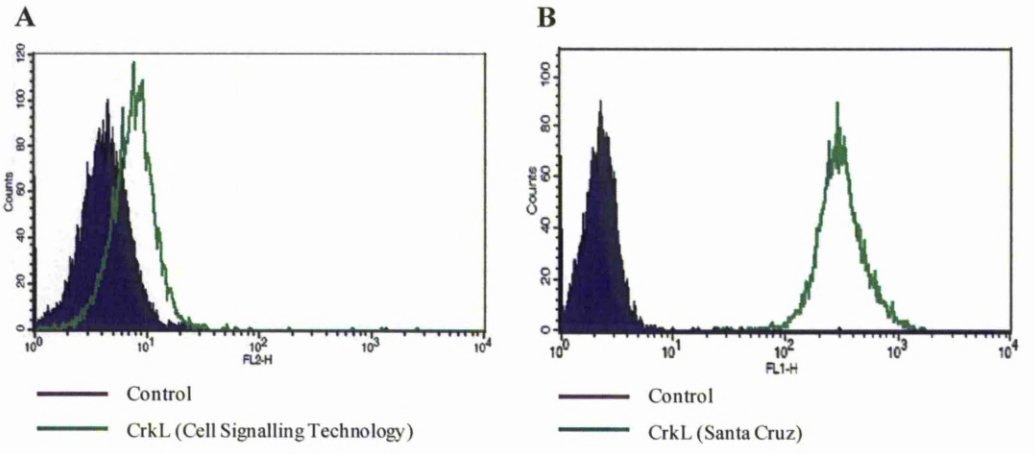


The outcome of different fixation and permeabilisation methods for the detection of pCrkL in K562 cells is shown in Figure 4.1. Fixation and permeabilisation were done using either the Caltag fix and perm kit following manufacturers instruction (panel A) or by our 'in house protocol' (panel B). Comparison of the resultant mean fluorescent intensity (MFI) shows that when the cells are fixed using the Caltag kit the change in MFI was 3.10. This increased to 12.21 MFI when the cells were prepared using the 'in-house' protocol. These data suggest that our 'in house' protocol using paraformaldehyde and methanol produces a greater degree of detection compared to the Caltag kit this was therefore chosen as the method for fixation and permeabilisation and was used in all subsequent experiments.

At the time of commencing this work, there was no FACS based assay reported in the literature for the detection of CrkL. None of the available antibodies had been shown to

detect CrkL by FACS, therefore antibodies previously shown to detect CrkL via Western blotting were used in these experiments. The next set of experiments was performed to investigate if CrkL could be detected by flow cytometry, as the detection of both pCrkL and CrkL by FACS would allow the pCrkL/CrkL ratio to be explored in a quick, reliable and quantitative manner which would be ideal for clinical samples. To investigate whether CrkL could be detected by FACS the BCR-ABL1 positive K562 and BCR-ABL1 negative U937 cell lines were used. The cells were fixed and permeabilised as described in section 2.6.1 and then labelled with either Cell Signalling Technology or Santa Cruz CrkL antibodies. FACS analysis revealed that CrkL could be detected in both the BCR-ABL1 positive K562 cell line (Figure 4.2) and the BCR-ABL1 negative cell line U937 (data not shown). This was the first time that CrkL detection has been demonstrated using FACS. Both antibodies detected CrkL; however the Santa Cruz CrkL antibody gave a better peak shift compared to the Cell Signalling Technology antibody (296.37 MFI and 18.8 MFI respectively). The Santa Cruz antibody was therefore chosen for the detection of CrkL by FACS and was used in all subsequent experiments.

Figure 4.2. Comparing two CrkL antibodies
Panel A: CrkL antibody (Cell signalling) **Panel B:** CrkL (Santa Cruz).



4.2.1.2. Saturating antibody concentration

An important part of the optimisation process was to determine the saturating concentration for the pCrkL and CrkL antibodies. To assess the amount of pCrkL antibody required to saturate the cells a range of concentrations were used from 7 – 56µg/ml on K562 and U937 cell lines. The saturating pCrkL antibody concentration was determined as 28µg/ml, as at concentrations greater than this no further increase in peak shift (MFI) was observed. This correlates with the concentration suggested by Hamilton *et al.*[199] The saturating concentration for the CrkL antibody was investigated over a range of concentrations from 7–56µg/ml. The saturating concentration was coincidentally determined to be 28µg/ml (data not shown).

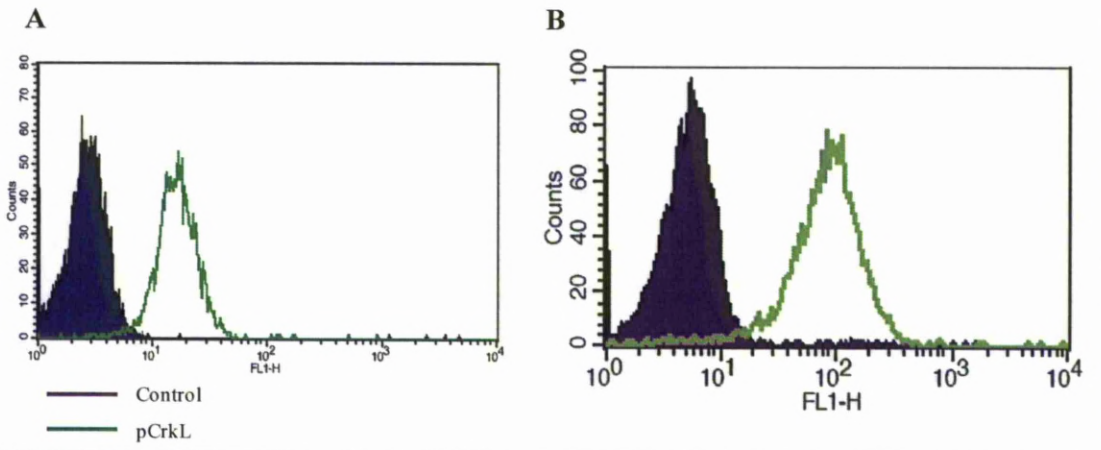
4.2.1.3. Triple layer FACS

Triple layer FACS techniques have been reported to enhance sensitivity by lowering the detection limit,[295] thereby enhancing the detection of molecules present at low levels. In an attempt to separate further the control and test peak, comparison was made using a triple layer FACS technique compared to the standard dual layer method. Results (not shown), indicated that there was no further increase in peak shift (determined by an increase in MFI) using a triple layer technique as compared to that observed with the dual layer technique.

4.2.1.4. Source of fluorophore

While this work was being undertaken Alexa Fluor dyes (Invitrogen) became commercially available. These fluorophores were marketed as being less labile, more photostable, pH-insensitive and with a superior fluorescence compared to more conventional fluorophores. In order to increase the peak shift further and hence the detection range of both pCrkL and CrkL, the use of Alexa Fluor fluorophores was investigated. Comparison was made between FITC conjugated antibody (BD) and their Alexa Fluor 488 counterparts. For the detection of pCrkL Alexa Fluor 488 was compared with a FITC conjugated antibody which was the antibody used by Hamilton *et al*[199] for the detection of pCrkL in CD34+ cells. Figure 4.3A shows the results achieved with the FITC conjugated antibody, demonstrating an MFI peak shift of 12.41. The peak shift observed for the Alexa Fluor 488 was 85.50, which was superior to that observed with the conventional FITC antibody (Figure 4.3B). For the detection of CrkL Alexa Fluor 488 was compared to a FITC antibody. The results were similar to those seen for the detection of pCrkL, with Alexa Fluor 488 enhancing the detection of CrkL by increasing the peak shift (data not shown). As a consequence the Alexa Fluor antibody was used as second layer for all subsequent experiments.

Figure 4.3. Comparison of second layer antibodies for the detection of pCrkL.
Panel A: pCrkL in K562 cells using a FITC conjugated antibody as second layer fluorophore.
Panel B: pCrkL in K562 cells using Alexa Fluor 488 as fluorophore.

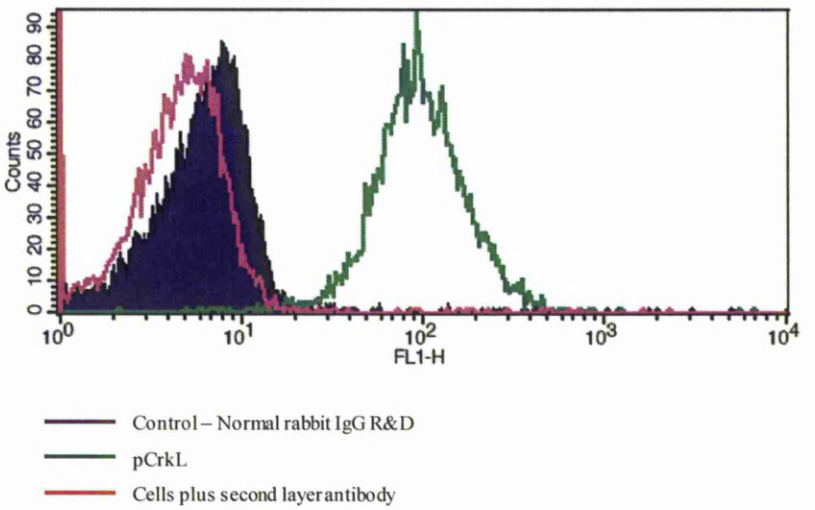


4.2.1.5. Source of control antibody

The pCrkL antibody is a polyclonal antibody which detects endogenous levels of CrkL only when it is phosphorylated at tyrosine 207. Hamilton *et al*[199] used cells plus the second layer antibody alone as their control for measuring pCrkL. The reasoning for this is that the pCrkL antibody is a polyclonal antibody and unlike monoclonal antibodies, it is not possible to use a class matched control. An improvement on the current published methodology might be to use a species specific total IgG or whole rabbit serum as a control. As part of the optimisation process two normal rabbit IgGs (R & D Systems and Santa Cruz) and two rabbit serums (Sigma and Abcam, Cambridge, UK) were investigated as controls. The aim was to compare different controls as a way of enhancing peak separation between the control and test peaks, and eliminating any false positivity due to non-specific binding. The controls were used at the same protein concentration as the pCrkL antibody (28µg/ml). All controls were tested on both the BCR-ABL1 positive (K562) and BCR-ABL1 negative cell lines (U937). The concentration matched normal rabbit IgG (Santa Cruz) control experiment revealed that this

reagent binds non-specifically, since the MFI value for the control is greater than that of the test antibody. The normal rabbit serum (Abcam), normal rabbit serum (Sigma-Aldrich) and normal rabbit IgG (R&D) gave MFI peak change values of 100.98, 109.74 and 103.04 respectively. These considerable peak shifts between the control and the pCrkL peak would allow a greater range of pCrkL to be detected; any of these three controls would be appropriate to use. Normal rabbit IgG (R&D) was selected for use in all future experiments; this was simply based on cost. Although Hamilton *et al*[199] failed to use an appropriate control, coincidently their 'second antibody only' control gave similar results in the present experiments to the more appropriate control (normal rabbit IgG) (Figure 4.4). This suggests that their results are still valid; however the technique developed in this chapter is more appropriate, has greater sensitivity and would thus allow a greater range of pCrkL to be detected.

Figure 4.4. Assessment of controls
Detection of pCrkL in BCR-ABL1 positive K562 cells in comparison to two control strategies; using normal rabbit IgGs (purple) and cells plus second layer antibody alone (pink).



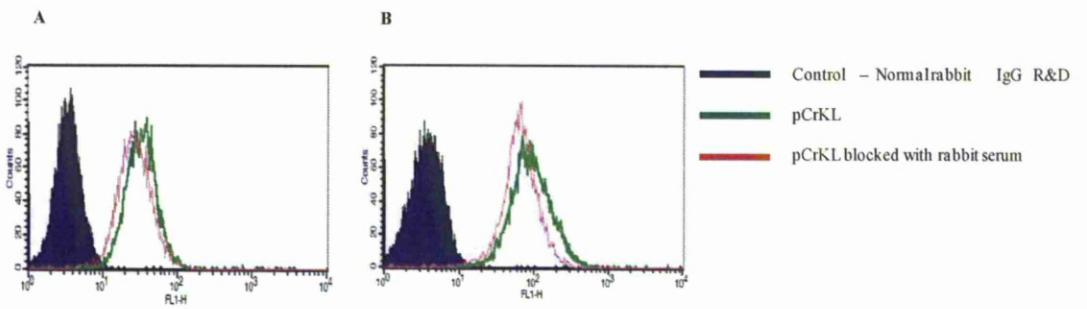
4.2.1.6 Fresh and frozen material

All the optimisation experiments were carried out using cell lines which have been continually growing in culture. Primary cells from our CML biobank containing frozen MNC were then assessed to see whether the results obtained from cultured cell lines were reproducible on frozen cells. Firstly, the pCrkL and CrkL levels measured in frozen material did not reflect those observed in the same sample if studied when freshly isolated. Secondly, levels of both pCrkL and CrkL varied and were not reproducible on different aliquots of the same frozen sample. It was therefore decided that all patient samples should be analysed using freshly isolated cells. Finally, it was determined that the minimum number of cells required to detect both pCrkL and CrkL in this protocol is 8×10^3 (data not shown).

4.2.2. Endogenous pCrkL

The BCR-ABL1 negative cell line U937 expresses low endogenous levels of pCrkL. To confirm that the endogenous pCrkL is not due to non-specific binding, both BCR-ABL1 positive and negative cells were pre-incubated with rabbit serum in order to block any non-specific binding sites. The pCrkL antibody was also pre-absorbed. The results from these experiments show that there is a small degree of non-specific binding but this is not responsible for the amount of pCrkL detected, hence confirming that endogenous pCrkL is present (Figure 4.5). The BCR-ABL1 positive cell lines KCL22 and K562 express high levels of pCrkL. It is therefore possible that CML cells express both endogenous and BCR-ABL1 mediated pCrkL. It is plausible that the BCR-ABL1 driven pCrkL responds to BCR-ABL1 targeted tyrosine kinase inhibitors but the endogenous pCrkL does not. The effects of TKIs on pCrkL will be discussed in section 4.2.6.

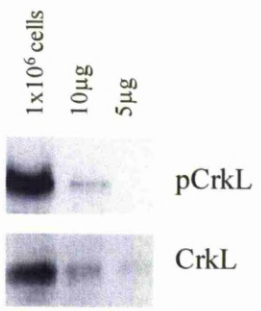
Figure 4.5. Endogenous pCrkL
Panel A: U937 BCR-ABL1 negative cells and **Panel B**: K562 cells, were pre-incubated with rabbit serum to prevent any non specific binding of the pCrkL antibody. This demonstrates that any pCrkL detected in BCR-ABL1 negative cells is due to endogenous pCrkL and not due to non specific binding of the antibody.



4.2.3. Detection of CrkL and pCrkL by Western Blotting

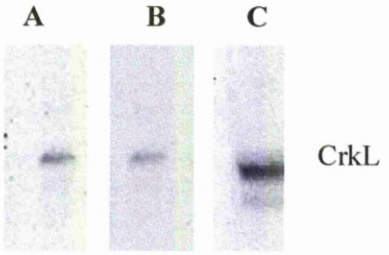
White *et al*[197] investigated the level of pCrkL in peripheral blood samples from patients with CML by Western blotting. Although this method is time consuming and requires a large number of cells it is useful for independently confirming the FACS results. A series of experiments were undertaken to establish a robust method for the detection of both pCrkL and CrkL in order to allow confirmation of the FACS results. Initially sample loading by protein concentration and cell number were compared. Western blotting was performed using a standard protocol, using pCrkL, CrkL antibodies (Cell Signalling Technology), and CrkL antibody (Santa Cruz). Figure 4.6 demonstrates the presence of pCrkL in the K562 cell line. pCrkL can be detected when either $1x10^6$ cells or 10µg of protein were loaded. pCrkL could not be detected when 5 µg of protein was loaded. CrkL could be detected at all concentrations loaded.

Figure 4.6. Western blotting demonstrating the presence of pCrkL
Western blot demonstrating the presence of pCrkL in K562 cells. Comparing loading by cell number to protein concentration. pCrkL cannot be detected in the sample loaded with 5µg of protein suggesting this concentration is too low.



Antibody specificity was confirmed by Western blotting by performing cross-over experiments. Briefly, two membranes were probed with different CrkL antibodies and then stripped and re-probed with the alternative antibody. The results confirmed that both antibodies were detecting proteins at the same molecular weight (Figure 4.7A and B). The same results were observed when the cross over experiment was performed using the antibodies in reverse order. The same experiment was also performed using the pCrkL antibody. CrkL was immunoprecipitated using the polyclonal CrkL antibody (Santa Cruz) and Western blotting was performed, probing the membrane using the monoclonal CrkL antibody (Cell signalling technology) (Figure 4.7C).

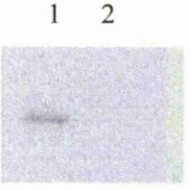
Figure 4.7. CrkL crossover experiment.
A: Western blot probed with monoclonal CrkL antibody (NEB). B: Membrane A stripped and re-probed with polyclonal CrkL antibody (Santa Cruz). C: CrkL immunoprecipitation.



4.2.4. pCrkL in non-malignant myeloid cells

CrkL has been identified as the most phosphorylated protein in neutrophils from patients with CML.[296] Hence it is important to determine if pCrkL can be detected in non-malignant myeloid cells. To achieve this, a neutrophilic blood sample was obtained (WBC 12.2 and neutrophils 9.2) from a patient who did not have CML. The results show that pCrkL could not be detected in non-malignant myeloid cells by Western blotting (Figure 4.8).

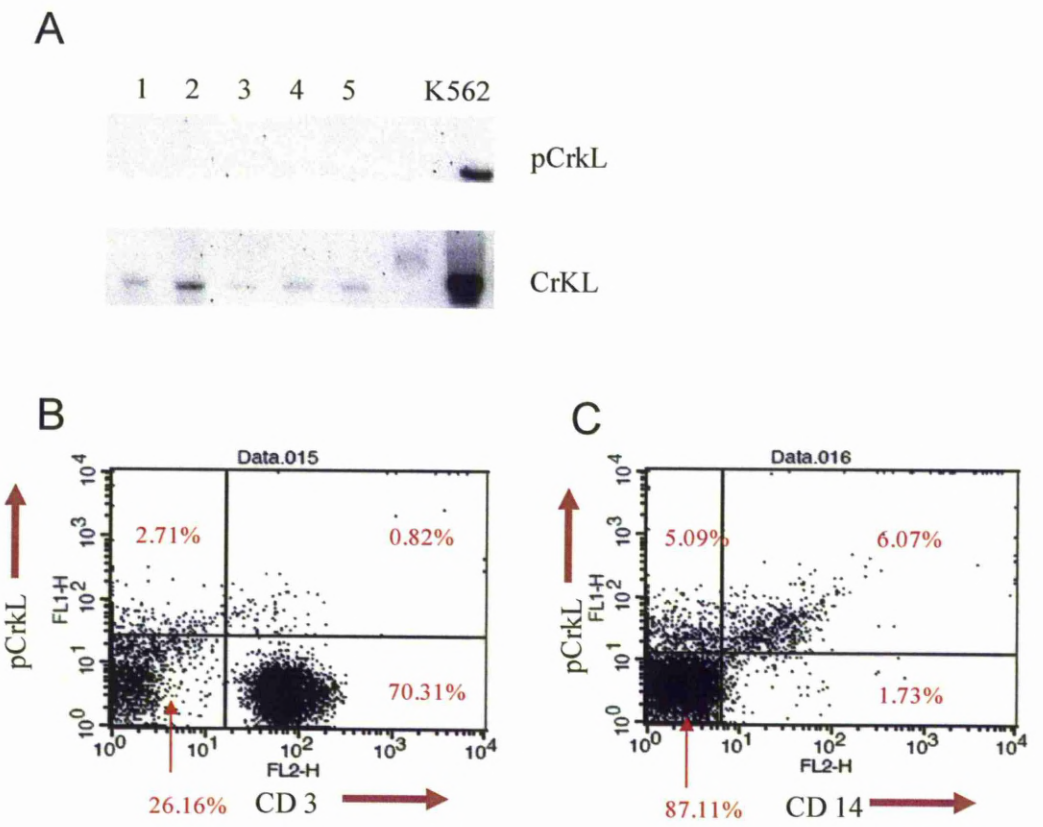
Figure 4.8. pCrkL cannot be detected in non-malignant myeloid cells.



4.2.5 pCrkL levels in normal blood mononuclear cells (MNC)

To ascertain the level of pCrkL and CrkL in normal blood, five healthy volunteers donated 20mls of peripheral blood from which MNC were collected. Western blotting and FACS were performed using the methodology given in section 2.6.1 and 2.7.0. No pCrkL could be detected in any of the five normal blood samples tested. CrkL could be detected in all samples (Figure 4.9A). FACS analysis demonstrated low levels of pCrkL in normal blood confirming that the FACS technique was more sensitive than Western blotting. FACS analysis of CrkL confirmed the Western blotting data with variable CrkL levels being detected. FACS analysis of normal samples dual labelled with pCrkL and CD3 (a lymphocyte marker; Figure 4.9B) demonstrated that the pCrkL signal observed was not attributable to the lymphocytes. CD14 co-staining showed that the pCrkL detected in normal MNC was largely within the monocyte (CD14 positive) cell population (Figure 4.9C).

Figure 4.9. pCrkL and CrkL levels in normal blood
Panel A: Western blot showing that pCrkL cannot be detected in MNC from five normal blood samples. CrkL protein is variable between samples. **Panel B:** FACS derived dot plots showing pCrkL levels in normal blood. The majority of CD3+ cells are pCrkL negative. **Panel C**: Illustrates that in normal blood monocytes are pCrkL positive.



4.2.5.1 Calculating the pCrkL/CrkL ratio

The pCrkL/CrkL ratio of a sample was determined thus:

$$\text{pCrkL/CrkL ratio} = \frac{\text{pCrkL- Control}}{\text{CrkL -Control}} \times 100$$

4.2.6. TKI treatment decreases the BCR-ABL1 tyrosine kinase activity.

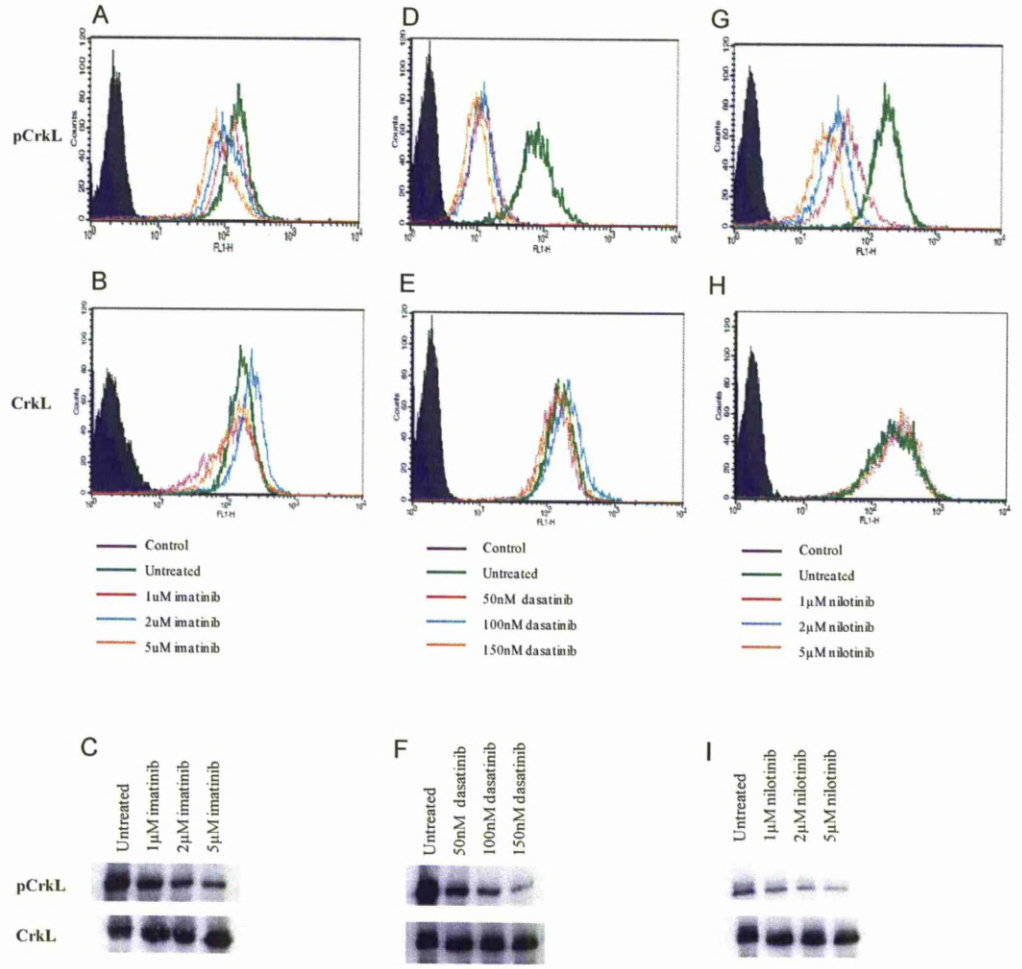
4.2.6.1 Imatinib treatment

To assess the inhibitory effects of imatinib treatment on CrkL phosphorylation, and the degree to which such changes are related to effects on BCR-ABL1 tyrosine kinase activity, K562 cells were incubated with varying concentrations of imatinib (1, 2 and 5μM) for 24 hours. Figure 4.10A shows that following imatinib treatment the K562 cells exhibit a dose dependent decrease in pCrkL levels. 5μM imatinib reduces pCrkL levels to 51.4% of the untreated drug control, with no effects on CrkL (Figure 4.10B). These results were confirmed via Western blotting (Figure 4.10C). Following imatinib treatment cell viability was assessed by flow cytometry using PI, indicating that 95% of the K562 cells were still viable.

4.2.6.2 Dasatinib treatment

In order to determine the effects of dasatinib treatment on pCrkL and CrkL, K562 cells were cultured for 24 hours with varying concentrations of dasatinib 50, 100 and 150nM (Figure 4.10D). The data suggest that dasatinib is more effective in suppressing CrkL phosphorylation compared to imatinib.

Figure 4.10. Effects of imatinib, dasatinib and nilotinib treatment on pCrkL and CrkL.
Panel A, D and G: pCrkL FACS plots. **Panel B, E and H:** CrkL FACS plots. **Panel C, F and I:** Western blots for pCrkL and CrkL for imatinib, dasatinib and nilotinib respectively.



4.2.6.3 Nilotinib treatment

K562 cells were treated with nilotinib at varying concentrations (1, 2 and 5μM) and the results show that nilotinib decreases pCrkL levels in a dose dependent manner, similar to that observed with imatinib. However, Figure 4.10G suggests that nilotinib is more effective at reducing CrkL phosphorylation compared to imatinib (89.9% and 48.6% reduction

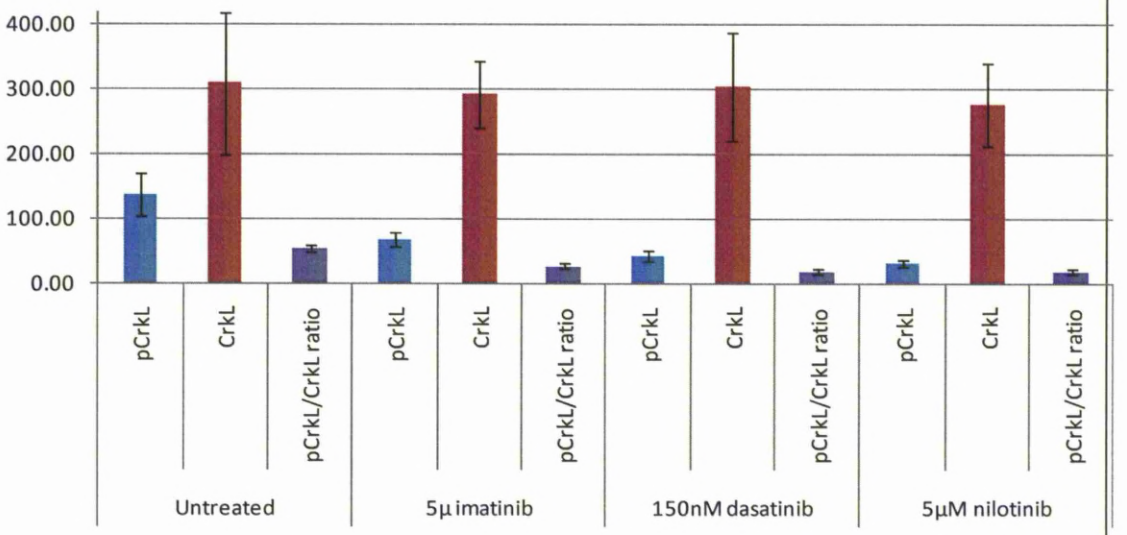
respectively). Dasatinib and nilotinib have comparable effects in suppressing pCrkL and hence BCR-ABL1 tyrosine kinase activity.

The drug concentrations used in the dose response curves are all pharmacologically achievable. For future experiments, the maximum pharmacologically achievable concentration of each drug was used (imatinib 5μM, dasatinib 150nM and nilotinib 5μM). Figure 4.11 compares the degree of BCR-ABL1 tyrosine kinase inhibition for imatinib, dasatinib and nilotinib by measuring changes in the pCrkL/CrkL ratio (n=9). Dasatinib and nilotinib both inhibit the pCrkL/CrkL ratio to virtually the same extent, with the % pCrkL/CrkL inhibition being 73.7% and 77.7% respectively. The pCrkL/CrkL ratio was inhibited by imatinib but to a lesser extent (58.8%). These results suggest that at typical concentrations achievable in patients, the second generation tyrosine kinase inhibitors are more effective than imatinib at inhibiting BCR-ABL1 tyrosine kinase activity. The suppression of pCrkL/CrkL ratio was statistically significant for all treatments compared to the untreated control ($p=<0.001$).

Figure 4.11. pCrkL/CrkL ratio decreases in K562 cells following treatment with TKIs

Panel A: Effects of imatinib (n=14), dasatinib (n=11) and nilotinib (n=10) on pCrkL, CrkL and pCrkL/CrkL ratio. **Panel B:** p values for changes in pCrkL and CrkL compared to untreated control.

A



B

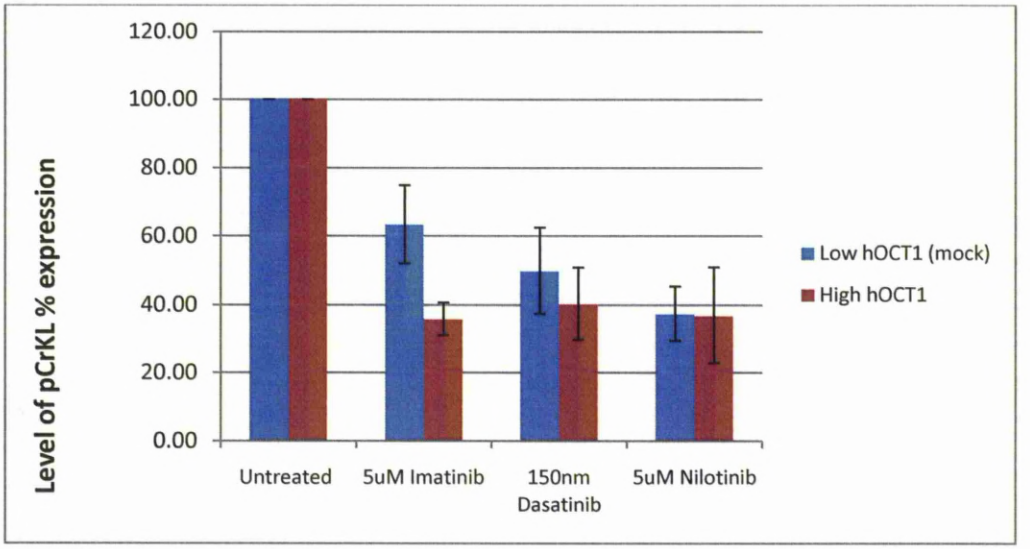
	pCrkL	pCrkL/CrkL ratio
Imatinib	p=0.074	p=<0.001
Dasatinib	p=0.015	p=<0.001
Nilotinib	p=0.027	p=<0.001

4.2.7. Efficacy and uptake of the second generation drugs

Our group has previously shown that imatinib is actively transported into CML cells via the influx transporter hOCT1,[165] and that the expression of hOCT1 is associated with clinical outcome following imatinib treatment.[166] To assess the efficacy and uptake of the second generation TKIs, their effects on BCR-ABL1 tyrosine kinase activity (pCrkL) were assessed by flow cytometry. The CML cell line KCL22 was selected for these experiments, since it expresses low basal levels of hOCT1.[297] KCL22 cells were transfected with hOCT1 to give a high hOCT1 cell line; mock transfected KCL22 were used as a control. These were produced by Dr Athina Giannoudis in our laboratory.

Dasatinib produced a significant decrease in pCrkL expression for both cell lines, with a reduction of pCrkL to 49.9% ($p=0.011$) in mock-transfected KCL22 cells and 40.3% ($p=0.003$) in high hOCT1-expressing cells, both in relation to untreated cells. Nilotinib also produced a significant decrease in pCrkL in both the hOCT1 high expressing and mock transfected cell lines (to 36.81% ($p=0.001$) and 37.35% ($p=0.001$) respectively; Figure 4.12). In contrast, imatinib reduced pCrkL to 39.7% of the control in high hOCT1-expressing cells ($p=0.001$), with no significant effect in mock-transfected cells. Comparison of hOCT1 and mock-transfected cells showed that the degree of pCrkL suppression by imatinib was greater in high hOCT1-expressing cells than in mock-transfected cells ($p=0.03$). These data are consistent with the view that hOCT1 transport is more important for imatinib than dasatinib or nilotinib.[147,298]

Figure 4.12. Effect of imatinib, dasatinib and nilotinib on BCR-ABL1 function. Effect of imatinib, dasatinib and nilotinib on pCrkL levels in mock-transfected KCL22 cells (blue bars) and KCL22 cells with high hOCT1 expression (red bars). Data are expressed as relative to untreated cells.



4.3.0. pCrkL, CrkL and pCrkL/CrkL ratio in CML patients

Having optimised and validated the pCrkL/CrkL assay as in sections 4.2.1 to 4.2.7, this was applied to investigate if the pCrkL/CrkL ratio at diagnosis was predictive of a patient's clinical response to imatinib treatment.

4.3.1. pCrkL, CrkL and pCrkL/CrkL ratio at diagnosis and clinical outcome.

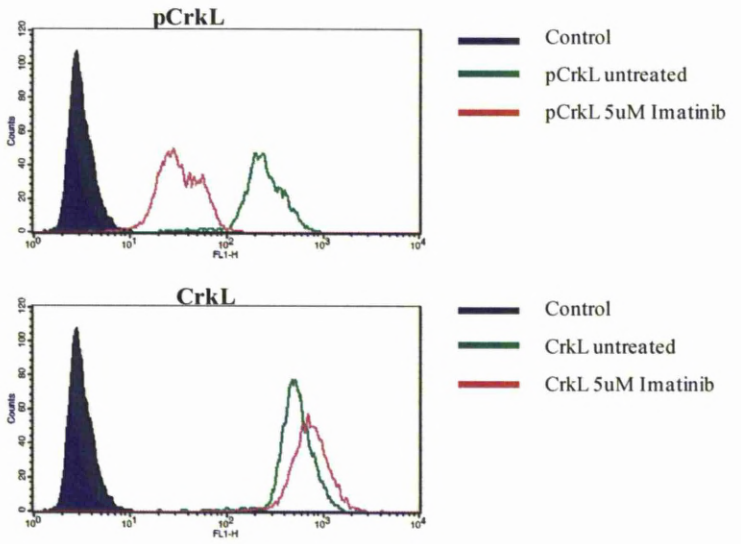
The 20 patients included in this study were all newly diagnosed chronic phase CML patients being treated at the Royal Liverpool University Hospital. All were 18 or more years of age at diagnosis, and were followed-up for a minimum of twelve months. Fourteen patients received imatinib 400mg daily and six patients received nilotinib 400mg daily as first line treatment. Patient characteristics are shown in Table 4.1.

Table 4.1. Summary of patients' characteristics

		Total	Imatinib treated	Nilotinib treated
Number of patients		20	14	6
Age in years (range)		48 (19-72)	46 (19-72)	54 (44-67)
Sex (M/F)		9/11	7/7	2/4
Sokal score	High	8	5	3
	Intermediate	6	3	3
	Low	6	6	0

MNC were analysed by flow cytometry for levels of pCrkL, CrkL and the pCrkL/CrkL ratio at diagnosis prior to receiving any treatment. Figure 4.13 shows representative FACS plots for pCrkL and CrkL pre and post *in vitro* imatinib treatment. These data show a suppression of BCR-ABL1 tyrosine kinase activity (pCrkL MFI) with only small changes in CrkL MFI.

Figure 4.13. Optimisation of pCrkL and CrkL detection in MNC by flow cytometry. Representative FACS plot showing pCrkL (upper plot) and CrkL (lower plot) in a newly diagnosed CML patient and changes following *in vitro* treatment with 5μM imatinib for 24 hours



pCrkL and the pCrkL/CrkL ratio were assessed at diagnosis prior to any treatment, and were then stratified by the patients' clinical outcome after 12 months of imatinib treatment (Figure 4.14). Levels of pCrkL alone (Figure 4.14A) do not correlate with clinical outcome, while the pCrkL/CrkL ratio assessed at diagnosis strongly correlates with the achievement of CCRe at 12 months ($p=0.0003$). Patients with a pCrkL/CrkL MFI ratio less than 25 prior to commencing treatment have a 75% chance of achieving a CCRe following 6 months of imatinib treatment, increasing to 100% at 12 months of imatinib treatment. In contrast, no

patient with a pCrkL/CrkL MFI ratio greater than 25 achieved a CCRe following 12 months of imatinib treatment (Figure 4.14B).

Figure 4.14. pCrkL/CrkL ratio assessed at diagnosis predicts a cytogenetic response.
Panel A: FACS assessment of pCrkL and Panel B: pCrkL/CrkL ratio in untreated newly diagnosed CML patients, stratified by clinical outcome following 12 months of imatinib treatment (n=14).

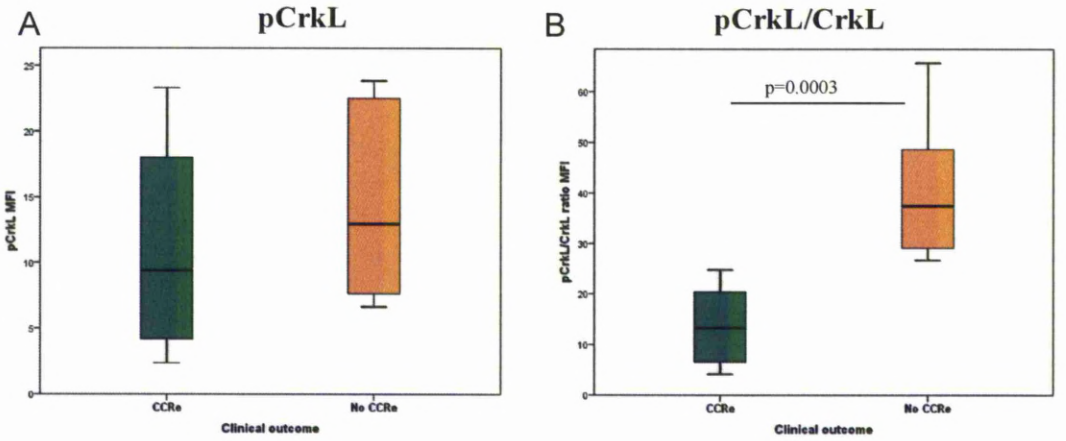
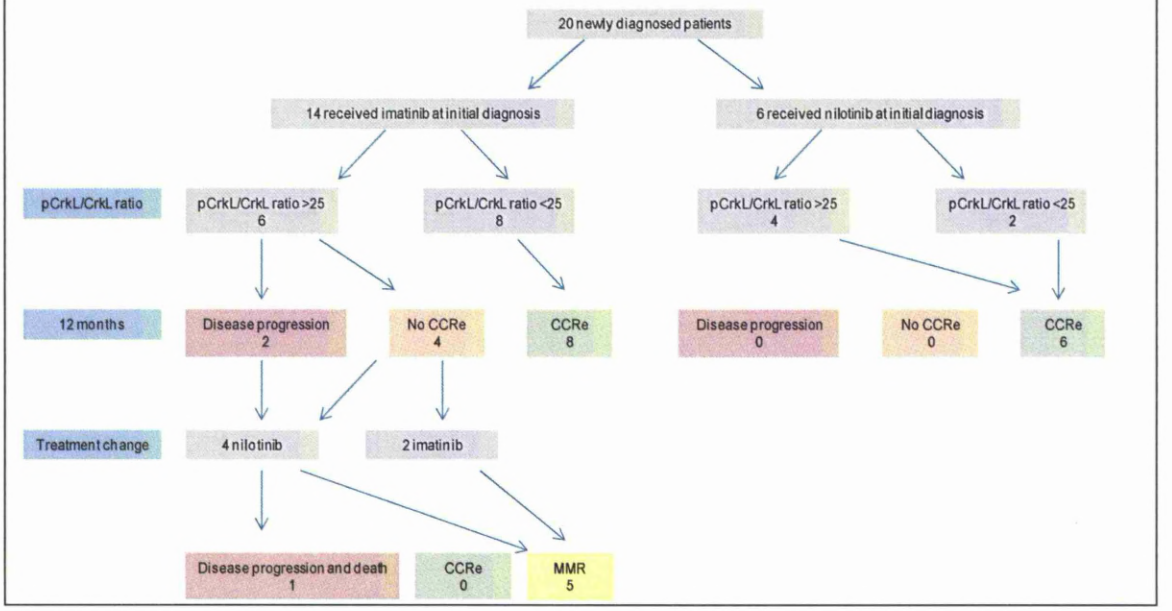


Figure 4.15. Clinical outcomes of patients in the pCrkL/CrkL study.



Six out of 14 imatinib treated patients investigated in this study failed to achieve a CCRe following 12 months of treatment, of whom two patients progressed to advanced phase disease. The clinical outcome of each patient is shown in Figure 4.15. Four of the six patients in the No-CCRe group switched to nilotinib treatment and three of these subsequently achieved a MMR or CMR. The single patient who failed to achieve a CCRe following nilotinib treatment also failed to achieve a response with dasatinib or chemotherapy and rapidly progressed into blast crisis which was fatal. The remaining two patients within the No-CCRe group continued on imatinib treatment but with dose modifications, and both subsequently achieved a MMR. No BCR-ABL1 kinase domain mutations were detected in these patients. The remaining eight patients, with a diagnostic pCrkL/CrkL ratio below 25 and a minimum follow-up of four years, all continue to respond well to treatment (one CCRe, three MMR and four CMR).

All patients with high pCrkL/CrkL ratios failed to achieve a CCRe following imatinib treatment, but following second line treatment with nilotinib, 75% of these went on to achieve a MMR or CMR. This suggests that the pCrkL/CrkL ratio in newly diagnosed chronic phase patients receiving nilotinib as first-line therapy. All six such patients treated with nilotinib achieved a CCRe (n=3) or MMR (n=3) following 12 months of treatment. With a minimum follow up of 30 months, 83% of patients have achieved a CMR. It is therefore not possible to show a comparison box-whisker plot for pCrkL and pCrkL/CrkL ratio for these patients since no patient failed nilotinib. Table 4.2 addresses this omission by tabulating the pCrkL/CrkL ratio for all patients in the study.

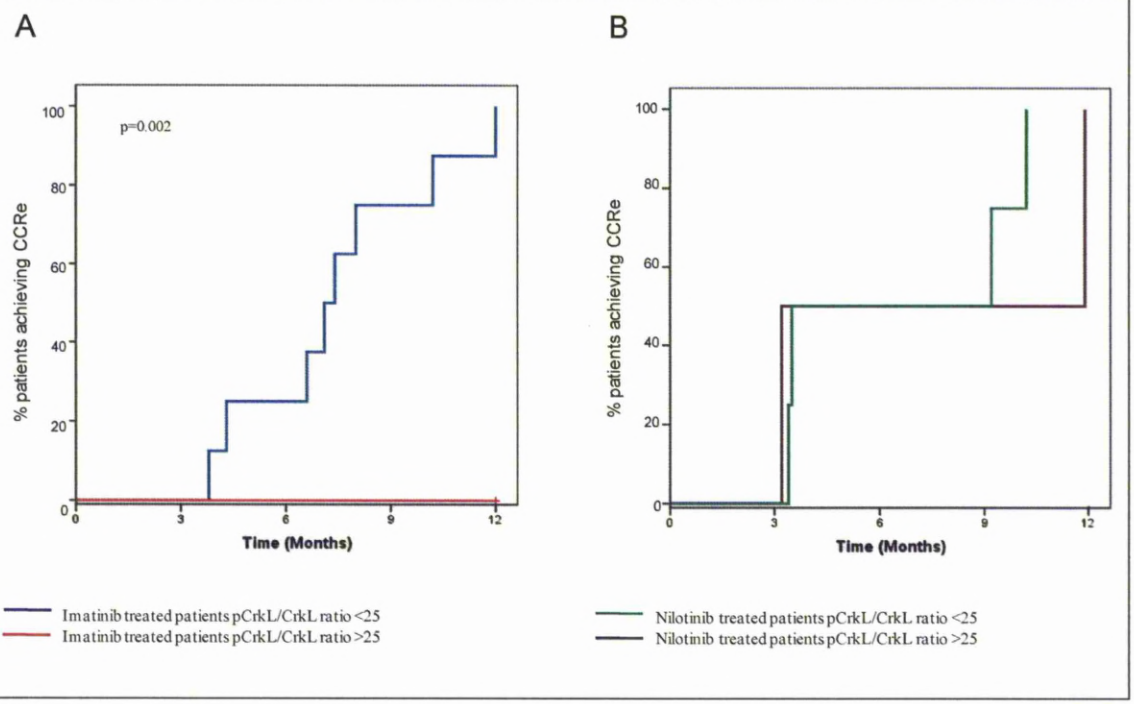
Table 4.2 pCrkL/CrkL ratios for imatinib and nilotinib treated patients.
pCrkL/CrkL ratios for imatinib and nilotinib treated patients along with their clinical outcome at 12 months. Patients with an MFI below 25 will all respond to imatinib treatment.

TKI treatment	pCrkL/CrkL ratio	CCRe at 12 months
Imatinib	4.13	Yes
Imatinib	6.05	Yes
Imatinib	7.01	Yes
Imatinib	11.67	Yes
Imatinib	14.77	Yes
Imatinib	16.19	Yes
Imatinib	24.5	Yes
Imatinib	24.74	Yes
Imatinib	26.68	No
Imatinib	29.13	No
Imatinib	35.29	No
Imatinib	39.57	No
Imatinib	48.62	No
Imatinib	65.70	No
Nilotinib	7.49	Yes
Nilotinib	12.31	Yes
Nilotinib	13.78	Yes
Nilotinib	18.71	Yes
Nilotinib	34.72	Yes
Nilotinib	47.3	Yes

Patients with a pCrkL/CrkL ratio greater than 25 who were treated with nilotinib achieved a CCRe compared to 0% of imatinib treated patients (p=0.03). This is most likely due to nilotinib being a more potent BCR-ABL1 tyrosine kinase inhibitor.[299]

Analysis of time to achieve a CCRe for imatinib and nilotinib treated patients stratified by pCrkL/CrkL ratio at diagnosis is shown in Figure 4.16. 100% of patients treated with imatinib and a pCrkL/CrkL ratio less than 25 achieved a CCRe, compared to 0% of patients with a pCrkL/CrkL ratio greater than 25. Irrespective of the pCrkL/CrkL ratio at diagnosis all nilotinib treated patients achieved a CCRe, although patients with a higher pCrkL/CrkL ratio took slightly longer to achieve a CCRe.

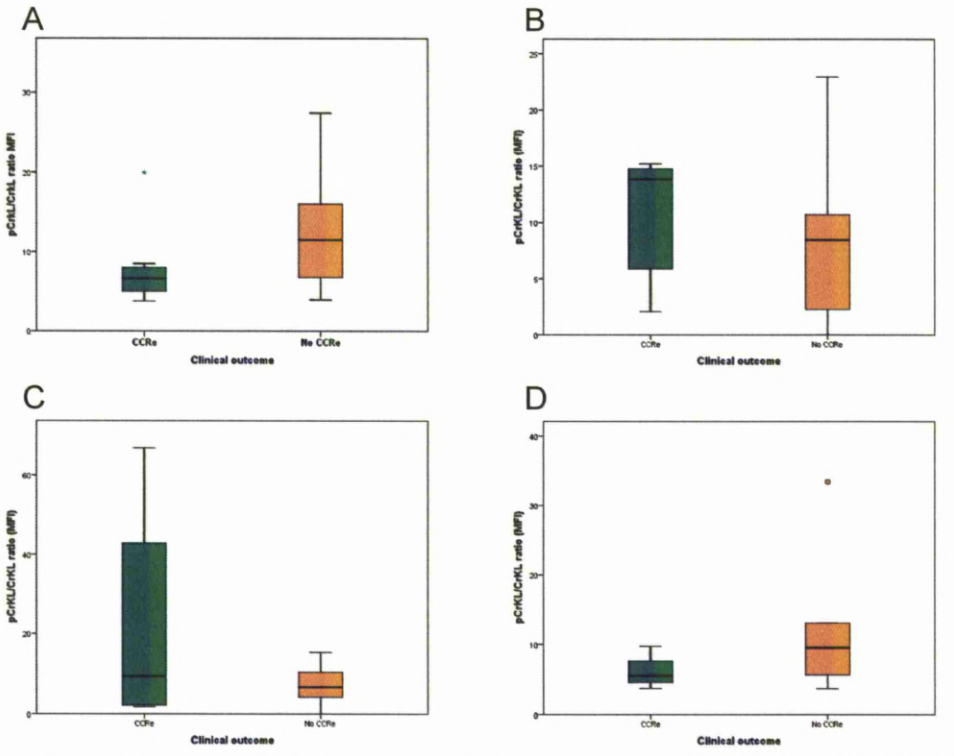
Figure 4.16. Time to achieve a CCRe
Kaplan-Meier estimate of time to achieve a CCRe for both imatinib (panel A) and nilotinib patients (panel B), stratified by pCrkL/CrkL ratio at diagnosis.



4.3.2. Measurement of pCrkL/CrkL ratio following *in vitro* treatment with imatinib

An assay was developed using MNC derived from untreated patients, to assess whether the *in vitro* effects of imatinib on BCR-ABL1 tyrosine kinase activity (pCrkL/CrkL ratio) were predictive of clinical outcome following *in vivo* imatinib treatment (Figure 4.17A). Following 24 hours of treatment with 5μM imatinib, samples from patients destined to achieve CCRe following 12 months of imatinib treatment had a similar pCrkL/CrkL ratio to those who failed to achieve a CCRe (p=0.491). These data demonstrate that an *in vitro* imatinib assay performed using freshly isolated MNCs does not reliably predict patients' clinical outcome.

Figure 4.17. Measurement of pCrkL/CrkL ratio following *in vitro* imatinib treatment and serial monitoring of pCrkL/CrkL ratio
Panel A: pCrkL/CrkL ratio in MNC from 14 newly diagnosed patients following treatment with 5μM imatinib for 24 hours, stratified by clinical outcome following 12 months of imatinib treatment. **Panel B, C and D:** serial monitoring of the pCrkL/CrkL ratio in samples (n=12) following 1, 3, and 6 months of imatinib treatment.



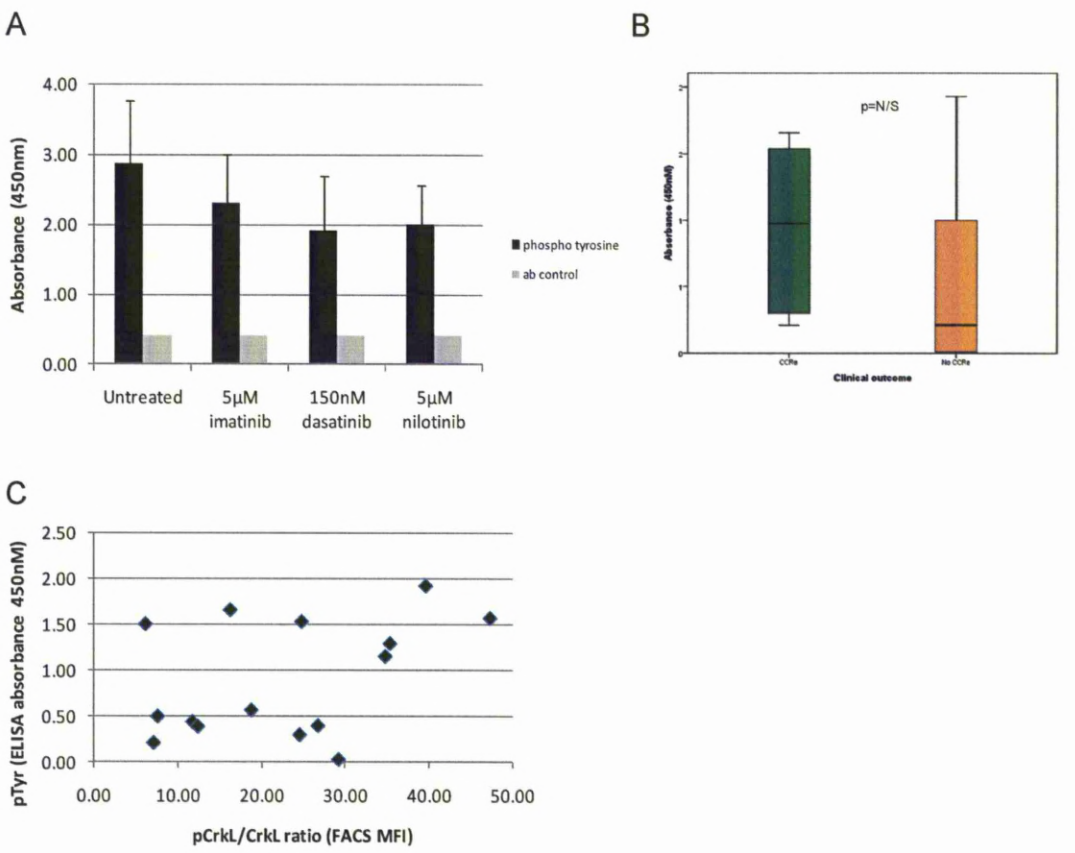
4.3.3. Serial monitoring of pCrkL/CrkL ratio following treatment with imatinib

Levels of pCrkL and CrkL were evaluated at 1, 3 and 6 months following commencement of imatinib treatment, using freshly isolated MNC at each time point. Although levels of both pCrkL and CrkL varied with time following treatment, these did not correlate with clinical outcome, thus the prognostic significance of the pCrkL/CrkL ratio is lost once treatment is initiated (Figure 4.17 B, C and D). These data suggest that measuring the pCrkL/CrkL at diagnosis is the critical time point for predicting which patients are likely to achieve a CCRe.

4.3.4 Tyrosine phosphorylation and clinical outcome

After developing the pCrkL/CrkL FACS assay, an ELISA method for the detection of phosphorylated tyrosine activity was published and the authors suggested that the results were comparable to their pCrkL FACS and Western blotting techniques.[294] This alternative method was tested initially using K562 cells. K562 cells were incubated for 24 hours with various TKIs at clinically achievable concentrations. These results show that following treatment with imatinib, dasatinib and nilotinib, phosphorylated tyrosine (p-Tyr) levels decreased (Figure 4.18A). Diagnostic samples from CP CML patients were then analysed and stratified by clinical outcome following 12 months of treatment. Figure 4.18B demonstrates that levels of p-Tyr at diagnosis of CML are not statistically significant between CCRe and No-CCRe groups. No correlation was observed between the pCrkL/CrkL ratio and p-Tyr levels assessed at diagnosis (Figure 4.18C).

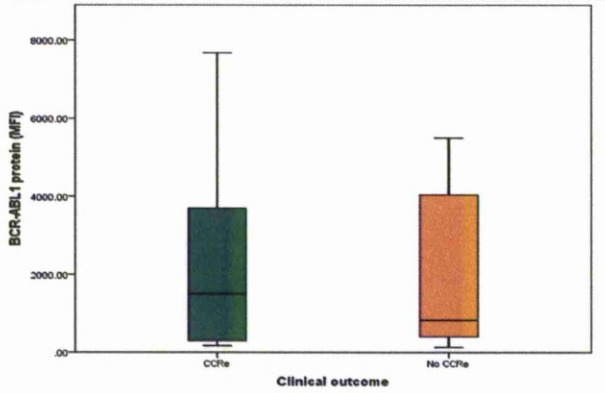
Figure 4.18. Total tyrosine phosphorylation ELISA.
Panel A: Assessment of phosphorylated tyrosine activity in K562 cells treated with imatinib, dasatinib and nilotinib. **Panel B:** Total tyrosine phosphorylation in CML MNC at diagnosis, stratified by the clinical outcome at 12 months (n=14). **Panel C:** No correlation was observed between pCrkL/CrkL ratio assessed by FACS and total tyrosine phosphorylation assessed by an ELISA in MNC at diagnosis.



4.3.5 Total BCR-ABL1 protein.

As discussed, it is difficult to assess the total amount of BCR-ABL1 protein in primary cells by conventional techniques such as Western blotting. Demonstrating BCR-ABL1 protein has been technically difficult up to now because of BCR-ABL1 protein instability,[189] and the lack of a specific antibody that can reliably distinguish BCR-ABL1 protein from its normal ABL1 counterpart. The BCR-ABL1 Protein kit (BD) has been developed to detect all frequently occurring BCR-ABL1 fusion variants in human blood, including p190, p210 and p230. The kit uses an antibody recognising BCR attached to a capture bead and an ABL1-directed phycoerythrin (PE)–conjugated detection antibody. The presence of BCR-ABL1 protein results in a sandwich complex comprising of both the capture bead and the detection fluorophore, detectable by flow cytometry. The design of the capture and detection antibodies permits the detection of all BCR-ABL1 variants. Total BCR-ABL1 protein was assessed using the BD BCR-ABL1 protein kit.[300] No difference was observed between the diagnostic BCR-ABL1 protein level and response to imatinib treatment. These data suggest that it is the BCR-ABL1 tyrosine kinase activity, and not the total amount of BCR-ABL1 protein which influences a patient's response (Figure 4.19).

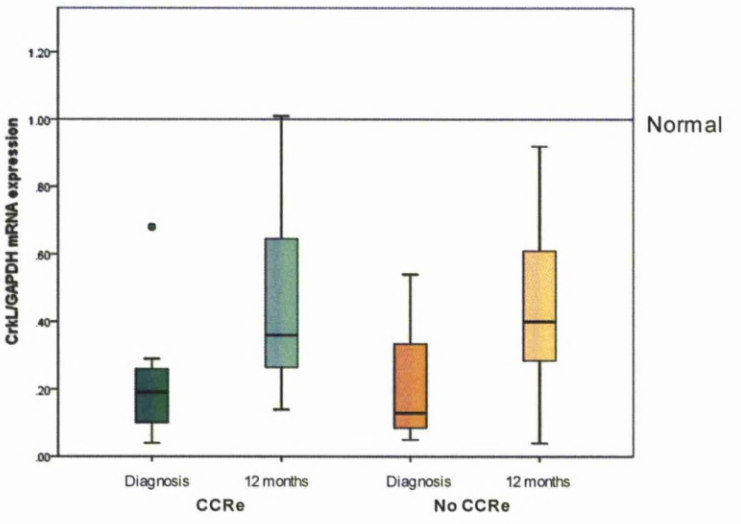
Figure 4.19. Total BCR-ABL1 protein.
Total BCR-ABL1 protein in untreated newly diagnosed CML patients, stratified by clinical outcome following 12 months of imatinib treatment (n=41)



4.3.6 CrkL mRNA expression.

CrkL mRNA gene expression was assessed at diagnosis and following 12 months of imatinib treatment. The data are normalised to a normal pool obtained from healthy volunteers. No difference in *CrkL* gene expression was observed at diagnosis or at 12 months for CCRe or No-CCRe patients (Figure 4.20). These data suggest that measuring *CrkL* mRNA expression offers no prognostic value.

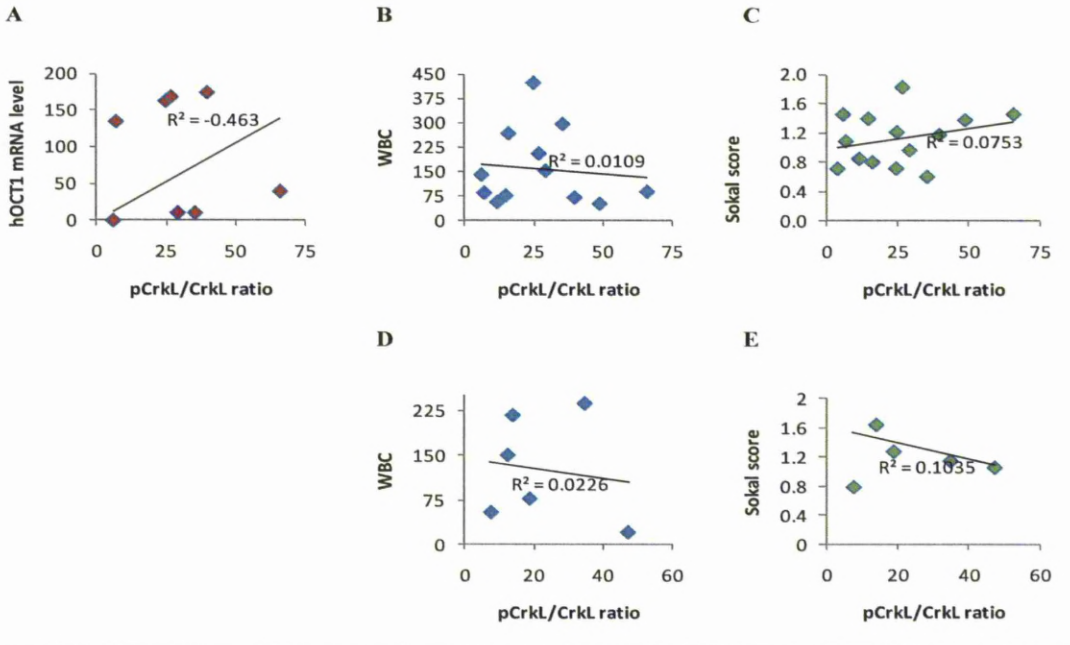
Figure 4.20. *CrkL* mRNA expression
CrkL mRNA expression was assessed at diagnosis and following 12 months of imatinib treatment, stratified by the eventual clinical outcome.



4.3.7. Relationship between the pCrkL/CrkL ratio and other prognostic parameters

Figure 4.21A demonstrates that there is no correlation between the pCrkL/CrkL ratio and hOCT1 expression in imatinib treated patients (n=8). hOCT1 gene expression was not assessed for nilotinib treated patients since hOCT1 is not an influx transporter for nilotinib.[298] Figure 4.21 B and D show that there is no correlation between the pCrkL/CrkL ratio and white blood count (WBC) at diagnosis. Figure 4.21C and E show that there is no correlation between Sokal score[301] and the pCrkL/CrkL ratio thus suggesting the pCrkL/CrkL ratio is an independent prognostic biomarker.

Figure 4.21. The pCrkL/CrkL ratio and other clinical parameters
Correlation between the pCrkL/CrkL ratio at diagnosis and A) hOCT1 mRNA level. B) white blood count (WBC) at diagnosis C) Sokal score for imatinib treated patients. Lower panels show correlation between the pCrkL/CrkL ratio at diagnosis and D) WBC at diagnosis E) Sokal score for nilotinib treated patients.



4.4.0. DISCUSSION

A considerable amount of time and effort was involved in the optimisation and validation of the pCrkL and CrkL FACS assays. The resultant pCrkL/CrkL assay is now a quick and simple assay which is both robust and accurate. Its potential applications within both the clinical and research fields are vast. To date it has been used independently and to support six publications from our research group.[94,147,298,302-304]

Imatinib is a tyrosine kinase inhibitor which has become the treatment of choice for newly diagnosed patients in chronic phase CML. In chapter three I have shown that in a population based study[21] by 24 months 49% of patients will fail imatinib treatment; similar findings were recently reported in two single centre studies.[287,290] This suggests that the identification of prognostic markers predictive of treatment response may be useful in order to avoid delay in offering alternative treatment such as a second generation TKI or SCT.

In this chapter I have developed a novel FACS based assay for the detection of both pCrkL and CrkL. Within this study it has been shown that measurement of the pCrkL/CrkL ratio at diagnosis by FACS is predictive of achievement of a CCRe following 12 months of imatinib treatment. 100% of patients with an MFI of less than 25 achieved a CCRe ($p=0.003$). Additionally, the pCrkL/CrkL ratio is not only predictive of clinical outcome at 12 months but also CCRe at six months, achieved in 75% of patients with an MFI of less than 25. All patients who achieved a CCRe remained in CCRe or have a deeper response such as MMR or CMR with a minimum follow-up of four years. No patient with an MFI above 25 achieved a CCRe following imatinib treatment; all subsequently required alterations in therapy which were either dose escalation or switching to an alternative TKI. No BCR-ABL1 domain

mutations were detected. Furthermore, serial monitoring of pCrkL, CrkL and the pCrkL/CrkL ratio following commencement of imatinib treatment was found not to be predictive of long term outcome. All patients treated with nilotinib at initial diagnosis all achieved a CCRe or MMR irrespective of their pCrkL/CrkL ratio suggesting that since nilotinib is a more potent TKI than imatinib it is therefore able to suppress higher tyrosine kinase activity to a greater extent.

Khorashad *et al*[203] reported that measurement of pCrkL levels alone by FACS did not correlate with clinical outcome.[203] The authors suggest a number of reasons for this discrepancy including sample sizes, imatinib affecting different cell populations to varying degrees and resistance being due to pathways independent of BCR-ABL1 kinase activity. In this study, measuring pCrkL in isolation either at diagnosis or following *in vitro* imatinib treatment supports their findings. Moreover, in the early stages of the optimisation process it became apparent that pCrkL and CrkL levels obtained from frozen MNC were not reproducible or reliable, and differed from those obtained from fresh cells, therefore all data presented here were obtained using freshly isolated MNC. The decision to use only freshly isolated cells slowed down this project considerably, firstly because it meant that it would not be possible to used samples stored in the CML biobank and secondly because samples had to be analysed at diagnosis and then the patient had to receive a minimum of 12 months of treatment before the data could be stratified for clinical response.

No correlation was observed between the pCrkL/CrkL ratio and presenting WBC, Sokal score or hOCT1 mRNA expression levels. This suggests that the pCrkL/CrkL ratio is an independent biomarker of clinical outcome. It is interesting to speculate that the response to

imatinib therapy is related to BCR-ABL1 tyrosine kinase activity present at diagnosis. A given dose of imatinib may therefore suppress the lower kinase activity to a greater proportional extent than the higher kinase levels in patients with an MFI above 25. This may explain the higher incidence of response following treatment with imatinib in the low pCrkL/CrkL group of patients. During this study six additional patients commenced nilotinib treatment at initial diagnosis. The pCrkL/CrkL MFI ratios ranged from 7.5 to 47.3. Following 12 months of treatment all achieved a CCR or MMR. These data suggest that patients with high BCR-ABL1 tyrosine kinase activity (pCrkL/CrkL MFI ratio >25) have a low probability of responding to imatinib, but may nevertheless achieve a CCR on nilotinib treatment.

In conclusion, the pCrkL/CrkL ratio prior to commencement of treatment is a potent predictor of clinical outcome in patients treated with imatinib. Its role as a predictive biomarker of response to TKI therapy merits further investigation in larger series, though it is essential that this is performed on freshly isolated cells prior to any treatment.

CHAPTER FIVE - Chronic myeloid leukaemia patients with the e13a2 bcr-abl1 fusion transcript have inferior responses to imatinib than e14a2 patients

5.1.0 INTRODUCTION

CML is characterised by a reciprocal translocation between chromosomes 9 and 22, which creates the fusion gene *BCR-ABL1*. Different types of *BCR-ABL1* transcript can be detected due to differences in the breakpoint. e13a2 and e14a2 are the most frequent *BCR-ABL1* transcript types in CML. The e13a2 and e14a2 *BCR-ABL1* transcript types differ in length by 75bp (25 amino acids).[37] Both *BCR-ABL1* mRNA molecules encode a 210kDa constitutively active protein kinase which is central to the pathogenesis of the disease as discussed in the general introduction section 1.3.3.[47]

Previous reports prior to the introduction of imatinib have in general not identified an effect of *BCR-ABL1* transcript type on clinical outcome.[54-60] In the imatinib era, one small study of 22 patients at different phases of disease suggested that the e13a2 *BCR-ABL1* transcript type may be more sensitive to imatinib treatment;[61] while a larger study has indicated that patients with e14a2 have a better molecular response to imatinib.[62] The clinical significance of the *BCR-ABL1* transcript type in newly diagnosed chronic phase CML patients treated with imatinib remains uncertain. In this chapter I assess if *BCR-ABL1* transcript type correlates with the clinical outcome by investigating the clinical outcome of 79 newly diagnosed patients with chronic phase CML treated with imatinib 400mg.

5.2.0 PATIENT COHORT

All patients (n=79) aged 16 or over with chronic phase CML newly diagnosed between January 1st 2003 and October 31st 2007 receiving imatinib 400mg daily from original diagnosis (preceded only by up to six weeks of hydroxycarbamide) and with a minimum of 12 months follow up were included in this study. The 71 patients who presented with either the e13a2 or e14a2 *BCR-ABL1* transcript type are the subject of the main investigation; patients presenting with both e13a2 and e14a2 transcript are discussed separately (three cases). Patients expressing rare transcript types were excluded from the main study (five cases; two cases of e19a2, and one case each of e6a2, e13a3, and e14a3 transcript) and will be discussed separately in detail on a case by case basis at the end of this chapter, leaving 71 cases expressing either e13a2 or e14a2 *BCR-ABL1* transcript types. Of these 32 presented with the *BCR-ABL1* transcript type e13a2 and 39 presented with the e14a2 transcript type. Age, sex and Sokal score of all analysed cases are presented in Table 5.1.

Table 5.1. Patient characteristics and response rates.

		All patients	e13a2	e14a2
Number of patients		71	32	39
Age (years)		50 (19-81)	48 (19-75)	51 (19-81)
Sex (M/F)		36/35	19/13	17/22
Sokal score	High	21	9	12
	Intermediate	14	5	9
	Low	15	6	9
	No data	21	12	9

5.3.0 RESULTS

5.3.1. Patients expressing e13a2 have inferior responses to imatinib than e14a2 patients.

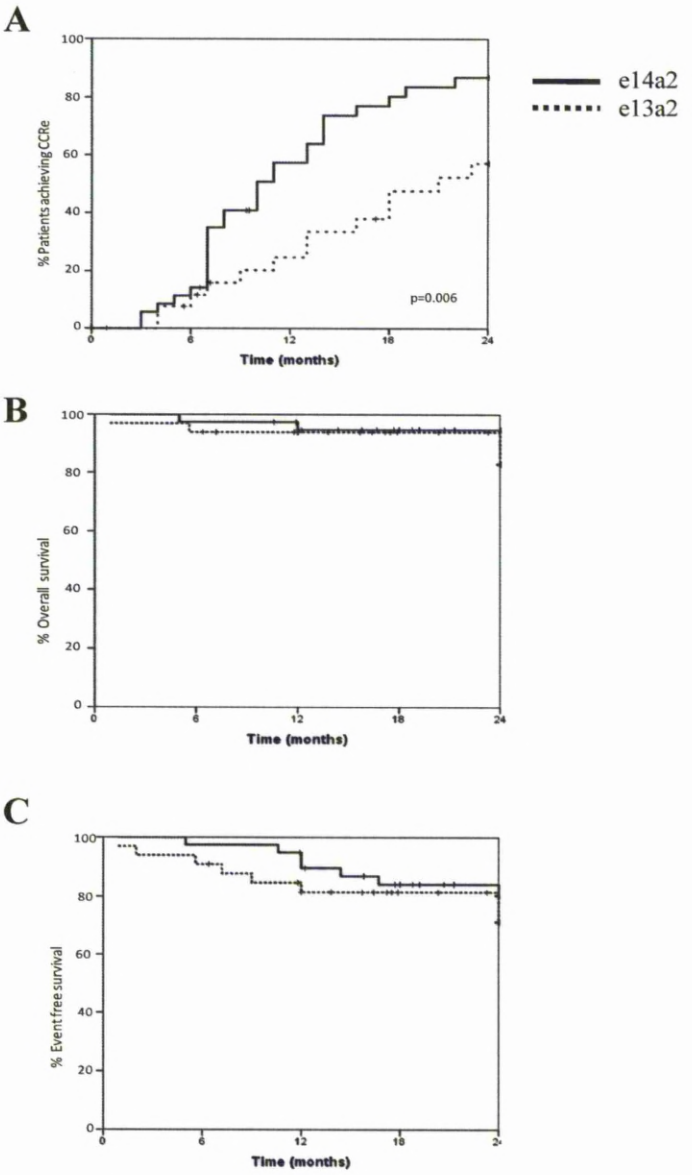
Following 12 months of imatinib treatment, 25% of patients with the e13a2 transcript achieved a CCRe compared to 54% of e14a2 patients (p=0.01, Table 5.2). At 18 and 24 months the e13a2 patients continue to achieve lower rates of CCRe compared to e14a2 patients. Kaplan-Meier analysis of time to achieve CCRe revealed that the patients with the e14a2 transcript type demonstrated more rapid response rates compared to e13a2 patients (Figure 5.1A), which continues throughout treatment (p=0.006).

Table 5.2. Patients response rates
CCRe rates for both e13a2 and e14a2 BCR-ABL1 transcript types at 12, 18 and 24 months*

		12 months			18 months			24 months		
		Total patients	CCRe	% of total patients achieving CCRe	Total patients	CCRe	% of total patients achieving CCRe	Total patients	CCRe	% of total patients achieving CCRe
Transcript type	**e13a2**	32	8	25.0	23	8	34.8	23	9	39.1
	e14a2	39	21	53.8	32	18	56.3	26	15	57.7

*At 18 months nine cases of e13a2 and seven cases of e14a2 are excluded from analysis as not yet having received 18 months of imatinib treatment. At 24 months six cases of e14a2 are excluded for the same reason.

Figure 5.1. Kaplan-Meier estimates of time to achieve CCRe, overall survival and event free survival.
Panel A: Kaplan–Meier estimate of time to achieve CCRe for e14a2 and e13a2 BCR-ABL1 transcript type. (p=0.006). **Panel B:** Overall survival **Panel C:** Event-free survival, stratified by BCR-ABL1 transcript type. No statistically significant differences were observed (SPSS statistical package–Mantel-Cox log-rank test).



Patients with the e14a2 transcript type demonstrated a non-significant trend toward superior EFS in the first 12 months of treatment, though overall survival did not differ significantly

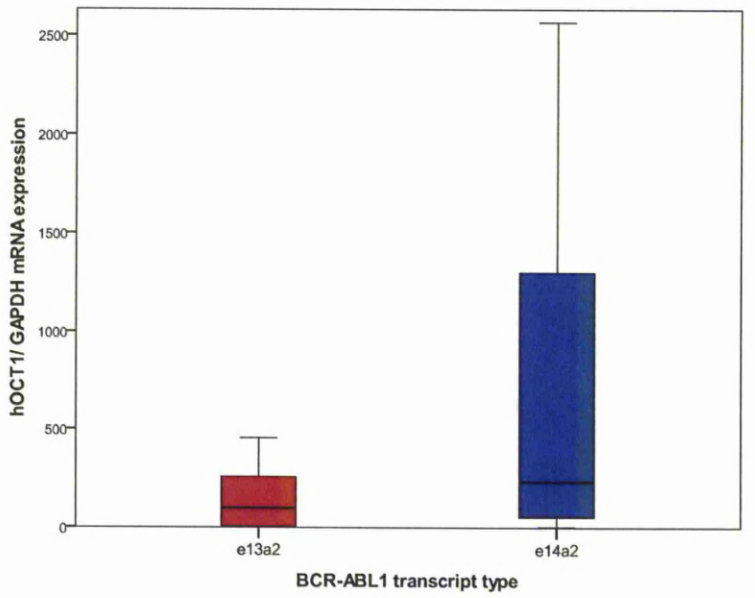
between the transcript types (Figure 5.1 B and C). This trend is consistent with the finding that more e13a2 cases progress in the first 12 months of treatment; eight e13a2 cases failed imatinib treatment (seven due to disease progression and one case was intolerance), while only three e14a2 cases failed (two disease progression and one intolerance). The Sokal score was not predictive of clinical outcome nor did it differ according to transcript type (Table 5.1). Of interest five additional cases (outside this study) who presented with blast crisis CML during the period of study all expressed the e13a2 transcript type.

Three patients presented with both e13a2 and e14a2 *BCR-ABL1* transcript types at diagnosis. Following 12 months of treatment, two achieved a CCRe, though by 24 months one of the cases subsequently lost their CCRe. The patient who failed to achieve a CCRe following imatinib treatment was switched to an alternative TKI but again failed to achieve a CCRe. Given the limited number of cases in this group it is not possible to form any definitive conclusion.

5.3.2 No correlation between BCR-ABL1 transcript type and the imatinib transporter hOCT1.

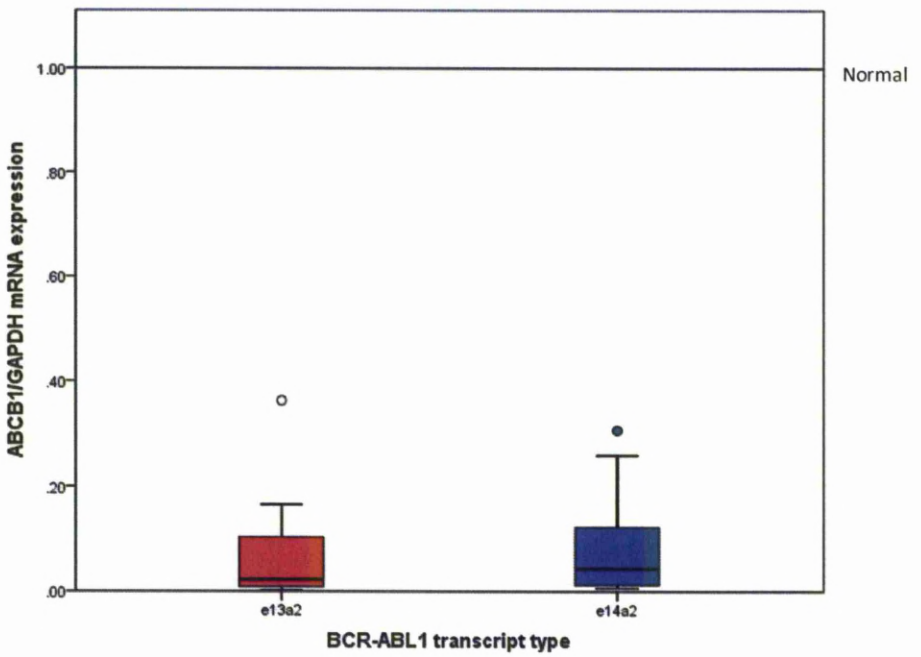
Imatinib uptake into CML cells is dependent on the uptake transporter hOCT1.[165] To establish why these two transcript types respond differently to imatinib treatment we determined hOCT1 mRNA levels in all patients for whom suitable material was available (n=51). No relationship was seen between transcript type and hOCT1 mRNA levels. The degree of imatinib uptake between the two transcript types was not statistically significant, although there was a trend for hOCT1 expression in e14a2 patients (Figure 5.2). (hOCT1 and ABCB1 mRNA expression studies were carried out by Dr Athina Giannoudis)

Figure 5.2. hOCT1 mRNA for e13a2 and e14a2 patients.
hOCT1 mRNA expression level was measured pre-treatment in 51 newly diagnosed CP CML patients. This demonstrates that e13a2 and e14a2 patients have similar levels of hOCT1.



The expression of the imatinib efflux transporter ABCB1 (MDR1) was assessed in all patients for whom suitable material was available (n=47). No correlation was observed between ABCB1 gene expression and BCR-ABL1 transcript type (Figure 5.3). In summary, these data demonstrate that patients with the e13a2 transcript have an inferior response to imatinib treatment and that this was not related to different hOCT1 and /or ABCB1 gene expression.

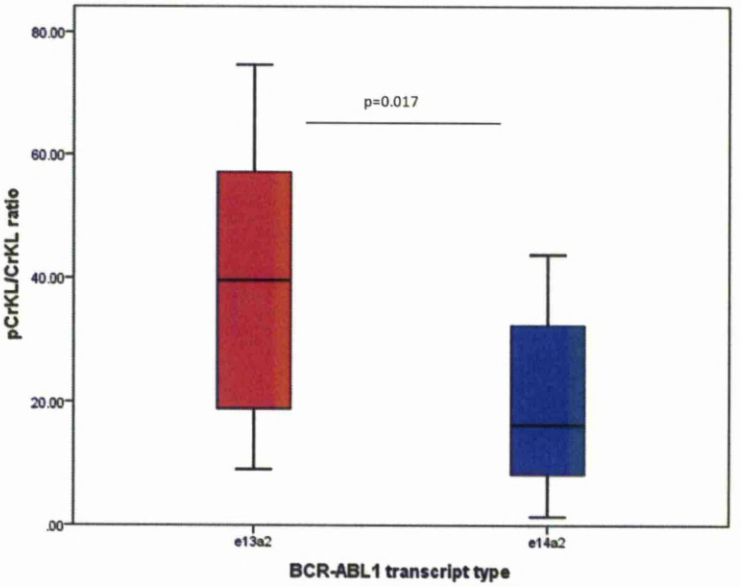
Figure 5.3. ABCB1 mRNA for e13a2 and e14a2 patients.
ABCB1 mRNA expression level was measured pre-treatment in 47 newly diagnosed CP CML patients. e13a2 and e14a2 patients have similar levels of ABCB1. ABCB1 expression is presented relative to a normal pool, which is shown as one on the graph below.



5.3.3 e13a2 transcript type patients have higher BCR-ABL1 kinase activity.

The pCrkL/CrkL ratio is a known surrogate marker for BCR-ABL1 tyrosine kinase activity.[197,303] To further investigate why these two transcript types respond differently to imatinib treatment the diagnostic pCrkL/CrkL ratio was determined in all patients for whom suitable material was available (n=28). These data demonstrate that samples from e13a2 patients have higher pCrkL/CrkL ratio than those from e14a2 patients (p=0.017), demonstrating higher tyrosine kinase activity (Figure 5.4). Since imatinib is a tyrosine kinase inhibitor, these data suggest that imatinib is only able to modestly suppress tyrosine kinase activity. Further supporting evidence was presented in chapter four, in which patients with a high diagnostic pCrkL/CrkL ratio failed to achieve a CCRe while on imatinib treatment, but when switched to the more potent TKI nilotinib, almost all achieved MMR. Moreover, newly diagnosed CP patients treated with nilotinib all achieve a CCRe irrespective of the diagnostic tyrosine kinase activity or BCR-ABL1 transcript type.

Figure 5.4. pCrkL/CrkL ratio for e13a2 and e14a2 patients
The pCrkL/CrkL ratio was measured pre-treatment in 28 newly diagnosed CP CML patients as a surrogate marker for BCR-ABL1 tyrosine kinase activity. This illustrates that e13a2 patients have a higher pCrkL/CrkL ratio than e14a2 patients (p=0.017).

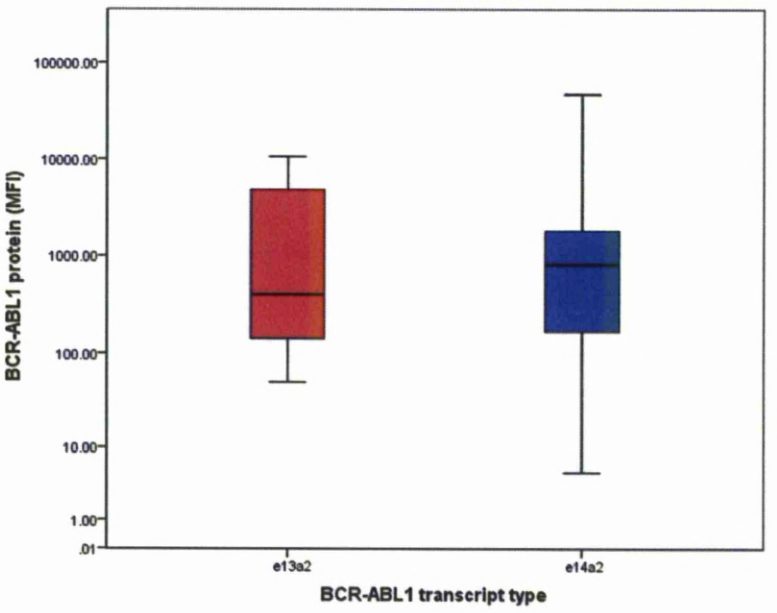


5.3.4 Does the total amount of BCR-ABL1 protein differ according to transcript type?

As discussed in Chapter four, it is difficult to assess the total amount of BCR-ABL1 protein in primary cells by conventional techniques such as Western blotting. Demonstrating BCR-ABL1 protein has been technically difficult up to now because of BCR-ABL1 protein instability,[189] and the lack of a specific antibody that can reliably distinguish BCR-ABL1 protein from its normal ABL1 counterpart. The BCR-ABL1 Protein kit (BD) has been developed to detect all frequently occurring BCR-ABL1 fusion variants in human blood, including p190, p210 and p230.[300] Utilising this kit, the total amount of BCR-ABL1 protein was assessed, to see if it differed between BCR-ABL1 transcript types (Figure 5.5). There

was no difference in the total amount of BCR-ABL1 protein between the two BCR-ABL1 transcript types tested.

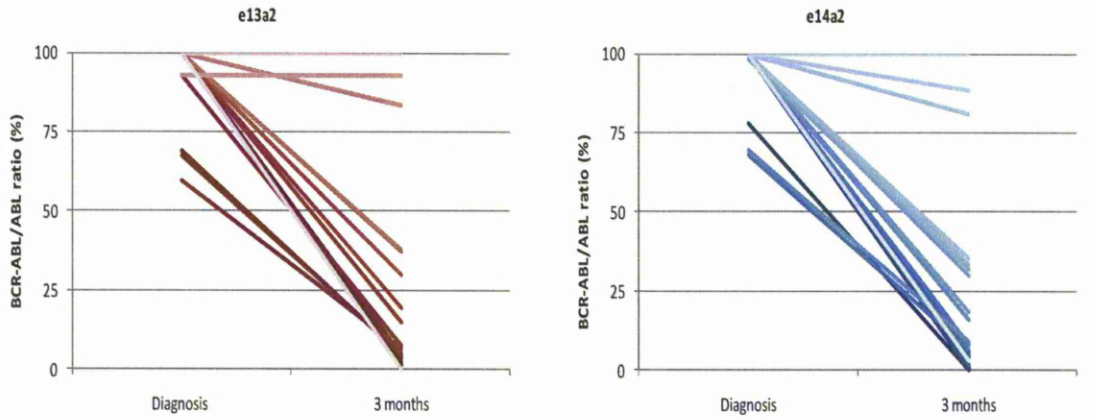
Figure 5.5. Analysing BCR-ABL1 protein in different BCR-ABL1 transcript types. Level of BCR-ABL1 protein stratified by BCR-ABL1 transcript type, e13a2 (n=26) and e14a2 (n=34).



5.3.5 The kinetics of response is not different between the two transcript types.

Previous work by our group has suggested that patients who have an early molecular response (defined as a BCR-ABL1 ratio below 10% at three months) have a significantly higher chance of achieving a CCRe and a better progression free survival.[163] It is therefore plausible that early changes in BCR-ABL1 ratio may be related to BCR-ABL1 transcript type. Figure 5.6 shows that there is no significant difference in the kinetics of response between the two transcript types (p=0.073).

Figure 5.6. Early changes in *BCR-ABL1* mRNA and BCR-ABL1 transcript type.
Changes in the BCR-ABL1 ratio between diagnosis and following three months of imatinib treatment according to BCR-ABL1 transcript type. No statistically significant difference was observed (p=0.073). Analysis was performed on all patients where a diagnostic and three month sample was available (n=51).



5.3.6 Variant BCR-ABL1 transcripts and clinical outcome.

The above data investigating the effects of *BCR-ABL1* transcript type on clinical outcome have demonstrated that patients expressing the e14a2 *BCR-ABL1* transcript type respond better to imatinib.[302] However, not surprisingly due to their rarity, few data are available on rare/variant transcripts types and their response to imatinib treatment. This section will focus on five patients with rare *BCR-ABL1* transcript types and their response to imatinib. None of the five patients described achieved a sustained CCRe on imatinib, and four died within 20 months of starting imatinib. Patient characteristics are displayed in Table 5.3.

Table 5.3. Variant transcript type patient characteristics.

Patient	Age /Sex	BCR-ABL1 transcript type	CML phase at diagnosis	First line therapy	Duration of imatinib treatment (months)	Achievement of CCRe	Clinical outcome	Overall survival
1	61/M	e19a2	Chronic	IFN	7	No	Blast crisis	Died
2	52/F	e19a2	Accelerated	IFN	48	No	Blast crisis	Died
3	65/M	e6a2	Blast crisis	imatinib	1.5	No	No response	Died
4	57/F	e13a3	Chronic	imatinib	18	Transient	Extramedullary relapse and T315I mutation	Died
5	59/M	e14a3	Chronic	imatinib	8	No	Switched to dastinib and currently in CMR	Alive

The e19a2 *BCR-ABL1* transcript type has been suggested to correlate with a more aggressive, unfavourable prognosis[305] and cytogenetic relapse has been reported when treated with imatinib, although another single case report suggested that e19a2 patients may exhibit a clinical course similar to typical CML.[306,307] Two patients presented here expressing e19a2 did not achieve a CCRe or a durable response while on imatinib.

The e6a2 *BCR-ABL1* transcript type has been reported to correlate with more aggressive disease and to be unresponsive to imatinib,[308] which is consistent with the findings here.

Reports of CML involving *ABL1* exon a3 instead of exon a2 fusing to BCR are rare. e13a3 has been reported to confer a more aggressive disease course[309] while others have reported that it responds well to imatinib.[310] Conversely there are currently no data about the e14a3 BCR-ABL1 transcript type and its response to imatinib therapy.

In general, data about rare BCR-ABL1 transcripts are sparse and mainly limited to single case reports. Attempts were made to investigate rare/variant transcript types from other UK institutions; however due to the rarity of these cases, there was a lack of samples with clinical data attached, making a large scale study not feasible.

5.4.0 DISCUSSION

In the pre-imatinib era, numerous studies addressed the significance of the BCR-ABL1 transcript type on CML, with conflicting results. The disparity can be explained by differences in sample size, sample selection, treatment and racial differences, as well as differences in statistical analysis.[55] Confusion can arise because some studies pooled e13a2 and e14a2 transcript types as 5' breakpoints; which were then compared to 3' breakpoints. These 3' breakpoints include e1a2 fusion transcripts that encode a p190 protein, which is associated with acute lymphoblastic leukaemia which has a poorer outcome than classical chronic phases CML. In general no difference in outcome of CP CML has been found in the pre-imatinib era between e13a2 and e14a2. However, surprisingly little data are available on the effects of these transcripts on response to imatinib treatment. This chapter has focused on comparisons between the e13a2 and e14a2 *BCR-ABL1* transcript types.

Studies in the pre-imatinib era suggest that the e13a2 junction may be more prevalent in men,[311] though not necessarily in all ethnic groups, and is associated with blast crisis of myeloid phenotype.[55] The e14a2 transcript type has been correlated with a higher platelet count both in adults[56,58] and children.[312] Additionally it has been associated with a longer chronic phase and survival, possibly related to the less aggressive course of chronic phase in this transcript type.[313] Furthermore, the e14a2 transcript type demonstrates a higher level of 5' *ABL1* deletions as an additional chromosomal abnormality, when compared to e13a2 patients.[55] Other reports found no correlations with the above, or with any other clinical or haematological parameters.[311,313-315]

Overall, data from the pre-imatinib era suggest that transcript type has no influence on clinical outcome.[54-60] However, only two series have addressed the effect of transcript type on the clinical outcome of imatinib treatment. One small study of 22 patients in Brazil with variable disease status reported a significant difference in response to treatment at six months (p=0.03), with e13a2 responding better than e14a2 patients.[61] A much larger study of 251 patients in CP treated with imatinib concluded that patients with e14a2 had a higher probability of achieving a major molecular response, and had a greater reduction in overall transcript levels in response to imatinib than e13a2 patients.[62] In spite of these interesting findings the authors offered no explanation as to why these transcript types respond differently to imatinib. Clearly the influence of transcript type on outcome following imatinib treatment warranted further investigation.

These data presented within this chapter support the view that patients with the e14a2 transcript may respond better to imatinib 400mg daily. Following 12 months of imatinib e14a2 patients not only achieve significantly higher CCRe rates than e13a2 patients but also achieve their CCRe at a faster rate. This was not related to the patients age, sex or Sokal score (which takes into consideration standard haematological parameters).[155] Additionally hOCT1 or ABCB1 mRNA expression did not differ between e13a2 and e14a2 patients; thus the degree of imatinib uptake and efflux between the two transcript types is similar and does not account for the differences in clinical responses observed. The reason for the difference in response to imatinib therapy observed therefore appears to be unrelated to differences in imatinib transport between transcript types. It is likely however that the effect is due to differences in the drug target. Using the pCrkL/CrkL ratio as an assessment of BCR-ABL1 tyrosine kinase activity,[197,303] e13a2 transcript type patients have a higher activity compared

to e14a2. A fixed dose of imatinib may therefore suppress the lower kinase activity in e14a2 patients to a greater proportional extent than the higher kinase levels in e13a2 patients. This may explain the higher incidence of CCRe following treatment with imatinib in e14a2 patients.

With respect to patients with variant *BCR-ABL1* transcript types, all patients failed to achieve a sustained CCRe on imatinib therapy, which is consistent with other similar reports. Four out of five patients reported in this study were diagnosed at a time when second generation tyrosine kinase inhibitors were not available at our institution. After failing imatinib treatment the patient expressing the e14a3 *BCR-ABL1* transcript type was switched to dasatinib and within six months had achieved a CCRe and MMR; she currently remains in CMR.

In this chapter I have shown that patients with e14a2 have lower intrinsic BCR-ABL1 tyrosine kinase activity at diagnosis and hence respond well to imatinib, while e13a2 patients with a higher BCR-ABL1 tyrosine kinase activity respond poorly. It is possible that the BCR-ABL1 tyrosine kinase activity in variant BCR-ABL1 transcripts is high thus resulting in a poor response to imatinib and a high incidence of disease progression; this hypothesis is supported by the data from the e14a3 patient who failed imatinib treatment but remains well on dasatinib (a more potent tyrosine kinase inhibitor). Alternatively varying breakpoints might change the tertiary structure of the BCR-ABL1 fusion protein which may alter protein: drug interactions.

Following the publication of the data of this chapter,[302] Suttorp *et al*[316] published a letter in Haematologica in response. The authors investigated the predictive value of *BCR-ABL1* transcript type on clinical outcome in 30 CP CML paediatric patients. Their study found that patients with the e14a2 transcript exhibited a more rapid decline in BCR-ABL1 mRNA and that this group of patients responded better to imatinib, confirming the findings within this chapter. Recently, at the EHA 2011 conference Castagnetti *et al*[317] analysed the effect of *BCR-ABL1* transcript type on behalf of the GIMEMA CML working party. The clinical outcomes of 493 patients were assessed. No significant differences in sex, age, Sokal/Hasford score, or clonal chromosomal abnormalities were observed between the two transcript types. No significant differences in CCR rates at 12 months were observed. However, time to achieve MMR was significantly shorter for patients with e14a2 transcript. The OS, PFS and EFS were superior for e14a2 patients. The authors suggested that the e13a2 transcript could be an adverse prognostic factor in CP CML patients treated with imatinib first-line.

In conclusion, patients with the *BCR-ABL1* transcript type e14a2 have a higher response rate to imatinib when the CCRe rate is compared to patients who express the e13a2 transcript type. This suggests that this may be due to e13a2 patients having a higher BCR-ABL1 tyrosine kinase activity. Knowledge of patient transcript type may yield clinically useful data, and should be included in future clinical trials of tyrosine kinase inhibitors.

CHAPTER SIX - The role of *ALOX5* in CML

6.1.0 INTRODUCTION

Malignancies are thought to be initiated by a single neoplastic cell, which is capable of self-replication; these cancer / leukaemic stem cells are thus the ideal therapeutic target. The role of *ALOX5* in the arachidonic acid pathway and CML is detailed more fully in section 1.7.0. Briefly, the *ALOX5* gene encodes a member of the lipoxygenase gene family and plays a role in the synthesis of leukotrienes from arachidonic acid. ALOX5 catalyses the conversion of arachidonic acid to 5-HEPTE, and further to LTA4. LTA4 is unstable and is converted to LTB4 which is more stable. 5-HEPTE and LTA4 negatively regulate *ALOX5* expression; positive regulation of *ALOX5* occurs when LTB4 binds to its receptor BLT1.

ALOX5 has been reported to be up-regulated in mouse LSC, and this up-regulation is not inhibited by imatinib. Furthermore, mice transplanted with *ALOX5* deficient BCR-ABL1+ bone marrow cells were resistant to the induction of CML.[253] *ALOX5* deficiency was found to have no effect on BCR-ABL1 negative cells. This suggests that *ALOX5* is important for mouse LSC growth and is essential for the development of CML in the mouse model studied.

The hypothesis proposed in this study was that CML cells have high levels of *ALOX5* and that the level of *ALOX5* predicts a patient's response to imatinib treatment. If this hypothesis were correct, then *ALOX5* may be a new therapeutic target. Zileuton is an *ALOX5* inhibitor which is currently licensed for the treatment of asthma. Zileuton alone or in combination with imatinib might be clinically beneficial in CML, since mice were resistant to the induction of CML when transplanted with *ALOX5* deficient BCR-ABL1+ bone marrow cells. The role of

ALOX5 in human CML is unknown. The aim of this chapter is to investigate the role of *ALOX5* in imatinib treated CML patients.

6.2.0. PATIENT COHORT

All patients (n=49) aged 16 or over with newly diagnosed chronic phase CML and with a minimum of 12 months follow up were included in this study.

Table 6.1. Patient characteristics

				Sokal score			
	No. patients	**M/F**	**Mean age (range)**	**Low**	**Intermediate**	**High**	**Unknown**
CCRe	25	11/14	43 (19-60)	15	3	5	2
No CCRe	17	8/9	49 (23-67)	4	3	7	3
BC	7	6/1	37 (24-59)	1	2	2	2
Total	49	25/24	44 (19-67)	20	8	14	7

Due to the specific sample requirement for this project, samples were not available from all patients for every experiment. For the study of *ALOX5* and *BLT1* expression in CD34+ cells, these were only available from three CCRe and three No-CCRe patients. For the ALOX5 protein assay, fresh peripheral blood was available from 12 patients. For the LTB4 ELISA, diagnostic plasma was available from 18 CCRe, 7 No-CCRe and 2 BC patients. For the assessment of the LTB4 receptor BLT1, fresh peripheral blood was used from 22 patients.

6.3.0 RESULTS

6.3.1 *ALOX5* mRNA expression and clinical outcome

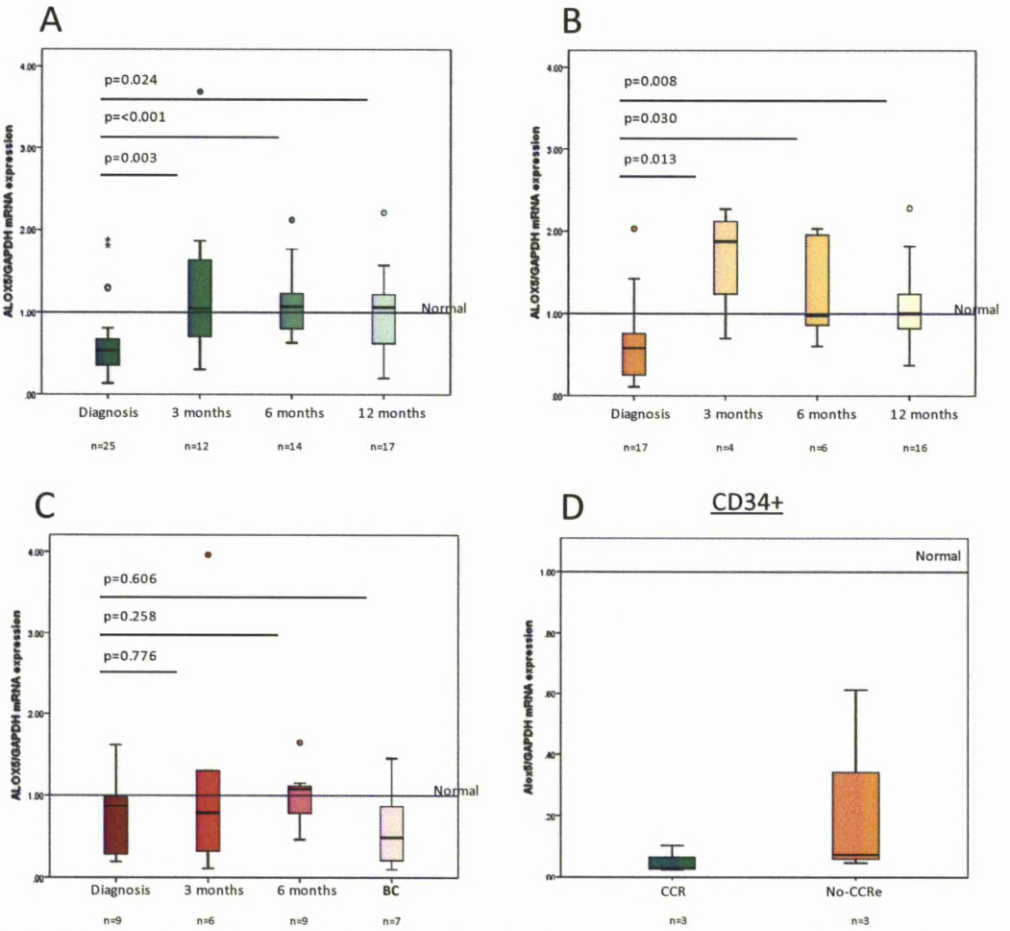
In order to determine whether *ALOX5* expression was also elevated in CML patients, and if the levels were related to related to clinical outcome, *ALOX5* expression was measured at diagnosis and following 3, 6 and 12 months of imatinib treatment. This was also studied in samples from four healthy volunteers. At diagnosis the expression level of *ALOX5* was below the level observed in normal healthy volunteers. This was the case in patients destined to achieve CCRe, No-CCRe or to later progress to blast crisis, as previously defined (Figure 6.1). In patients destined to achieve a CCRe, once imatinib treatment had commenced, the expression level of *ALOX5* increased to normal levels and remained at the normal level throughout treatment. The increase in *ALOX5* expression compared with diagnosis was statistically significant at 3, 6 and 12 months ($p=0.003$, $p=<0.001$ and $p=0.024$ respectively). These data suggest that *ALOX5* expression in patients who subsequently achieve a CCRe is restored (Figure 6.1A). A similar trend in *ALOX5* expression was observed in the No-CCRe group (Figure 6.1B), with *ALOX5* expression increasing once treatment had commenced. Again the increase in *ALOX5* expression compared with diagnosis was statistically significant at 3, 6 and 12 months ($p=0.013$, $p=0.030$ and $p=0.008$ respectively). In patients destined to subsequently progress to BC, imatinib treatment failed to increase *ALOX5* expression and the level of expression remained below the normal level.

These data may suggest that a failure to increase *ALOX5* expression following three months of imatinib treatment may indicate a higher risk of disease progression (Figure 6.1C). No difference in *ALOX5* expression was observed at diagnosis between those patients destined to

progress to blast crisis and the CCRe and No-CCRe groups, suggesting that *ALOX5* expression at diagnosis is not predictive of the outcome of imatinib treated patients.

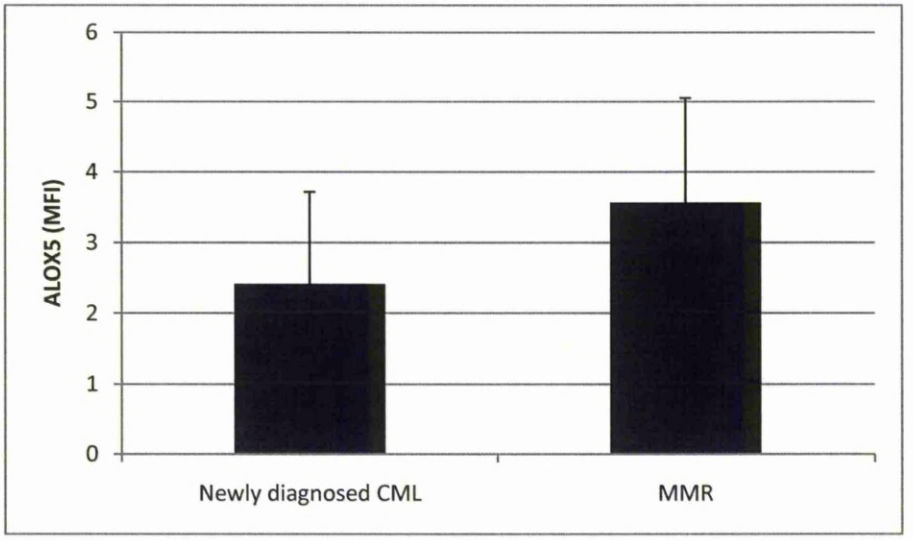
When the same experiment was repeated on diagnostic CD34+ cells (from the same patients), no difference in *ALOX5* expression was observed between responders and non-responders. *ALOX5* expression was lower in CD34+ cells than in total leukocytes, although similar overall results were obtained, confirming that *ALOX5* is down-regulated in CML patients in both CD34+ and mature cell compartments (Figure 6.1D).

Figure 6.1. *ALOX5* expression
Panel A: *ALOX5* expression levels in the CCRe group. **Panel B:** *ALOX5* expression levels in the No-CCRe group. **Panel C:** *ALOX5* expression levels in the BC group. **Panel D:** *ALOX5* expression levels in CD34+ cells taken at diagnosis and stratified by the patients' eventual clinical response. The normal level was determined using four healthy volunteers. Statistical analysis (Mann–Whitney U test) compared diagnostic values against 3, 6 and 12 month values.



To determine whether ALOX5 (5-LOX) protein levels were a function of *ALOX5* gene expression, ALOX5 protein expression was assessed by FACS. As patients respond to imatinib treatment and achieve a MMR, there was a trend for an increase in the amount of detectable ALOX5 protein (Figure 6.2); this is consistent with the increase in mRNA expression observed in Figure 6.1.

Figure 6.2 ALOX5 protein expression
ALOX5 protein was assessed by FACS using fresh leukocytes. ALOX5 expression is higher in CML patients with no detectable BCR-ABL1 as determined by qRT-PCR.



6.3.2 *ALOX5* function - LTB4 levels in CML patients

Plasma levels of LTB4 can be used as a marker of *ALOX5* functional activity (as described by Chen *et al*[253]). Preliminary work was carried out to determine the normal circulating level of LTB4 as well as the appropriate time point to measure the LTB4 levels in patients on treatment (Figure 6.3). LTB4 levels from 15 healthy volunteers with no signs of any

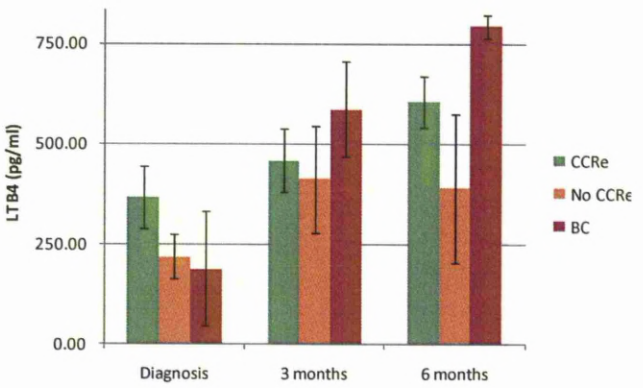
infection were assessed and the mean normal level was determined as 300pg/ml (data not shown). LTB4 levels were also investigated in plasma samples taken at diagnosis, 1, 3, 6, 9, and 12 months following imatinib treatment. The results suggested that measuring LTB4 at diagnosis, three and six months post imatinib treatment would show the greatest differences between optimal responders, failure patients and those destined to develop blast crisis.

LTB4 levels were measured at diagnosis, three and six months in 27 patients of known subsequent clinical outcome (18 CCRe, 7 No-CCRe and 2 BC) (Figure 6.3.A). LTB4 levels increased in all three patient groups following imatinib treatment. At diagnosis the LTB4 level in patients destined to progress to blast crisis (BC group) was the lowest of the three patient groups. Conversely, once imatinib treatment commenced the LTB4 level increased to the highest level compared to the other two clinical groups (Figure 6.3A). The levels of LTB4 in the CML patients were in general higher than normal; this may indicate an accumulation of LTB4 and a potential block in the arachidonic acid pathway (given in Figure 6.5).

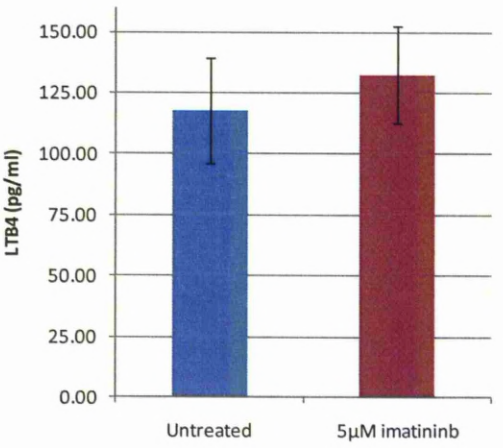
Since LTB4 levels increased in all patients assessed, it was necessary to confirm whether this was a direct physiological response to imatinib treatment, or if it was due to the disease. To address this issue, MNC from five healthy volunteers were treated with 5μM imatinib for 24 hours and the changes in LTB4 levels were measured. The data are shown in Figure 6.3B. No significant difference was observed between the untreated and imatinib treated normal samples, thus suggesting that changes in LTB4 observed in CML patients may be attributed to the leukaemia and not as a physiological response to imatinib treatment (Figure 6.3B). It was not possible to exclude continuous imatinib treatment as a cause for the increase in LTB4 (as this would have required healthy volunteers to take imatinib).

Figure 6.3. LTB4 levels following imatinib treatment.
A. Mean plasma LTB4 levels of 27 patients stratified by clinical outcome; 18 CCRe, 7 No-CCRe and 2 BC. Samples were taken during chronic phase at diagnosis, 3 and 6 months after commencement of imatinib treatment. **B**. Effects of 24 hours of 5μM imatinib treatment on LTB4 levels in five samples from healthy volunteers.

A



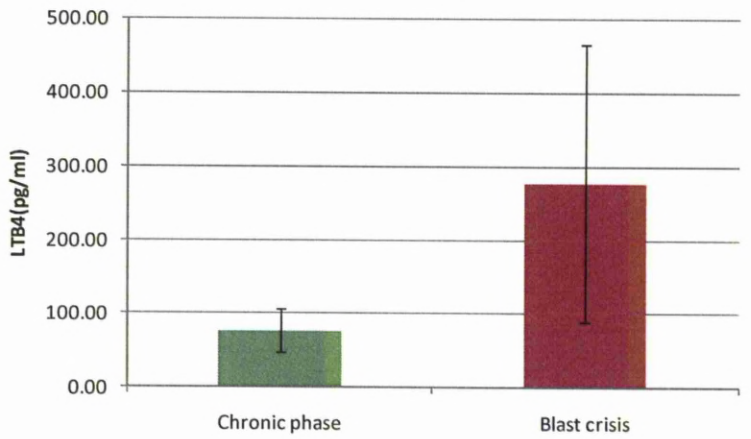
B



6.3.3 *ALOX5* function in chronic phase and blast crisis

ALOX5 function was assessed using samples from patients diagnosed in chronic phase and from patients actually in blast crisis (Figure 6.4). LTB4 levels were found to be elevated in samples taken at blast crisis compared to chronic phase samples, but this difference was not statistically significant. The increase in LTB4 observed coincided with a decrease in *ALOX5* expression.

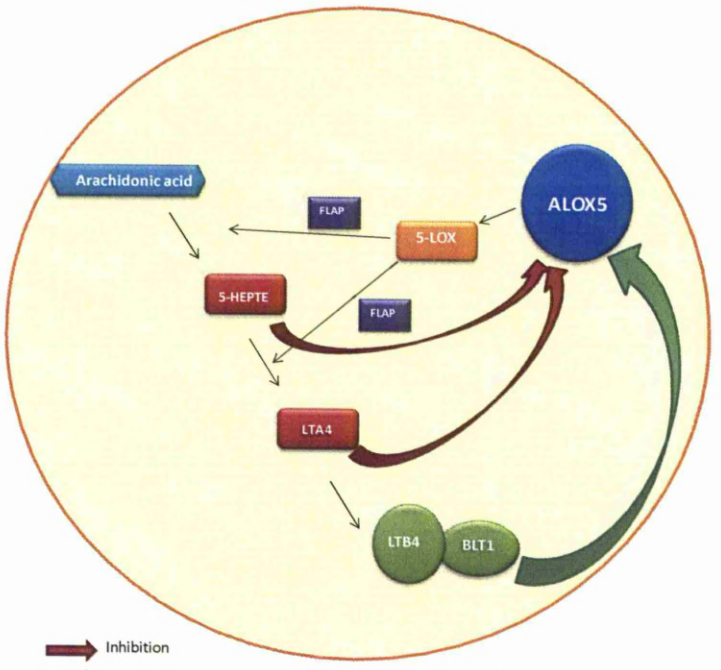
Figure 6.4. Comparing LTB4 levels in chronic phase with blast crisis.
Plasma LTB4 levels from chronic phase (n=27) and blast crisis patients (n=10).



6.3.4 LTB4 receptor BLT1

The original hypothesis proposed in this study was that CML patients would have high levels of *ALOX5*. The data presented thus far are in opposition to both this hypothesis and the study presented by Chen *et al*,[253] which demonstrated that mice transplanted with BCR-ABL1 positive bone marrow cells lacking the *ALOX5* gene were resistant to the induction of CML, suggesting that *ALOX5* was essential for the induction and development of CML.[253] However, that study was performed using a mouse model, and the experiments have not yet been carried out on human CML samples, which could explain the discrepancies between the present data and those of Chen *et al*.[253] In order to further explain the differences between the two studies, the LTB4 pathway was interrogated further. Figure 6.5 demonstrates the arachidonic acid metabolism pathway. Prior to conversion to LTB4, arachidonic acid is converted into two intermediate products, 5-HEPTE and then LTA4. Both 5-HEPTE and LTA4 negatively regulate *ALOX5* expression, while LTB4 is known to positively regulate *ALOX5* expression.[318] In order for LTB4 to mediate its positive effect on *ALOX5* expression it must bind to BLT1, the LTB4 receptor.

Figure 6.5. Arachidonic acid metabolism pathway.

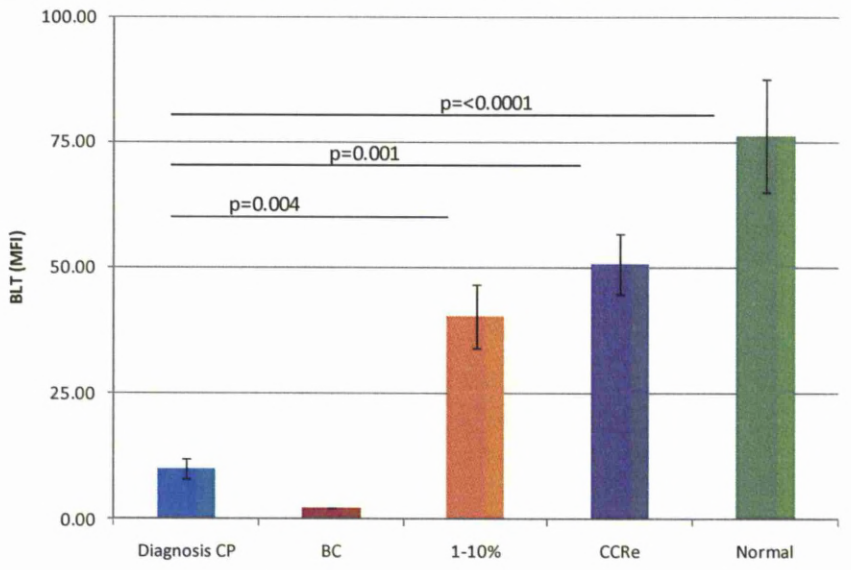


In the CML clinical samples investigated it is apparent that *ALOX5* expression is suppressed below normal. Furthermore, the LTB4 levels in the CML samples were elevated compared to normal healthy controls, suggesting an accumulation of LTB4. Since LTB4 needs to bind to its receptor BLT1 to positively regulate *ALOX5* expression, these data suggest that BLT1 expression or function may be abnormal in CML.

BLT1 protein expression was therefore measured by cell surface FACS analysis, and was found to be lower in MNC cells compared to a total leukocyte preparation. This is because LTB4 and hence *ALOX5* are important in neutrophils, which are lost in the preparation of MNC. This therefore meant that fresh blood samples were required to study BLT1 protein (rather than stored MNC preparations).

BLT1 surface expression was found to be very low in newly diagnosed CP CML samples (Figure 6.6). When the level of BLT1 was compared between newly diagnosed patients and patients responding to TKI treatment (defined as a BCR-ABL1 ratio of 1-10%), then BLT1 expression was significantly lower at diagnosis ($p=0.004$). When the comparison was made between BLT1 levels in newly diagnosed CML patients and either those in CCRe or in normal controls, again the difference was statistically significant ($p=0.001$ and $p=<0.0001$ respectively). These data suggest that there is an inverse relationship between BLT1 protein and BCR-ABL1 mRNA expression. BLT1 protein is very low at diagnosis but increases towards normal as the BCR-ABL1 mRNA expression decreases.

Figure 6.6. LTB4 receptor (BLT) protein level.
BLT1 surface expression was measured in newly diagnosed chronic phase CML patients (n=5), a patient in BC (n=1), patients currently responding to imatinib treatment (n=6), patients in CCRe (n=10) and healthy volunteers (n=15). BLT1 cell surface expression was found to be low in newly diagnosed chronic phase CML patients compared to patients responding to treatment or healthy volunteers.

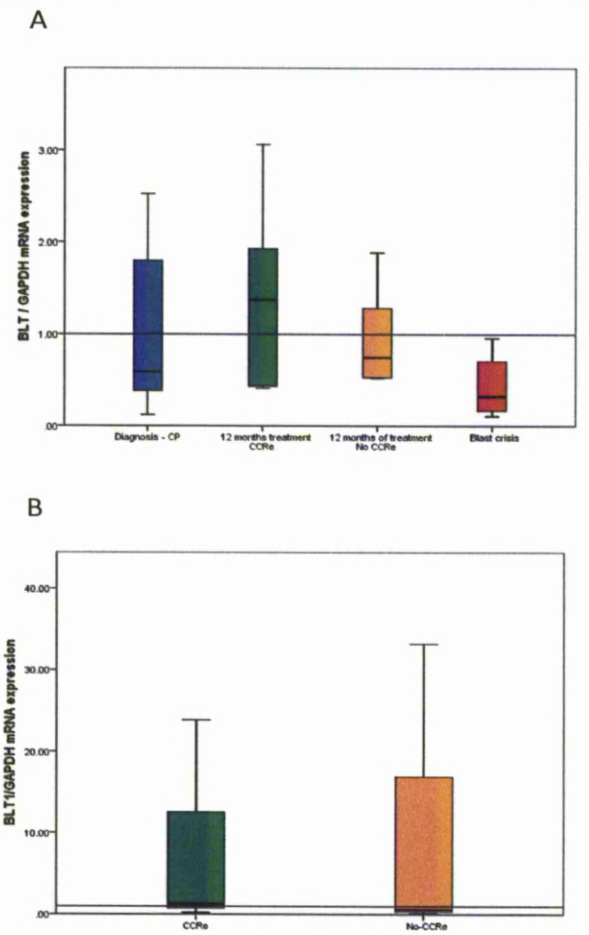


6.3.5 BLT1 mRNA expression

BLT1 mRNA expression was found to be lowest in CP diagnostic samples compared to patients who had received 12 months of imatinib treatment (Figure 6.7). In the CCRe group the BLT1 expression increased more than that observed in the No-CCRe group, although this was not statistically significant. When BLT1 mRNA expression was assessed in samples from patients in BC at the time of sampling, BLT1 mRNA expression was much lower than the diagnostic CP group and the 12 month samples. BLT1 mRNA expression was not statistically significant at diagnosis, nor did it predict a patient's clinical outcome (data not shown).

As patients progress to BC, *ALOX5* gene expression is suppressed, possibly due to an accumulation of pathway intermediates (5-HEPE and LTA4) and a decrease in BLT1. This idea is supported by the BLT1 protein data and the accumulation of LTB4 in blast crisis. BLT1 mRNA expression was also assessed in CD34+ cells taken at diagnosis (Figure 6.7B). No difference in BLT1 expression was observed between those patients who later achieved a CCRe and those who did not.

Figure 6.7. LTB4 receptor (BLT1) mRNA expression.
Panel A: BLT1 mRNA expression was measured in newly diagnosed chronic phase CML patients (n=24), patients treated with imatinib for twelve months who achieved a CCRe (n=10) and No-CCRe (n=8), as well as patients who were in BC (n=6). **Panel B:** BLT1 mRNA expression in diagnostic CD34+ cells stratified by the patient's clinical outcome.



6.4.0 DISCUSSION

The identification of proteins which discriminate leukaemic from normal stem cells is a challenging concept. Successful identification and therapeutic targeting of leukaemic stem cells remains the ultimate goal in eradicating CML.

The present data suggest that although *ALOX5* has been identified as being essential for the development of CML in mice, this is not the case in human CML. Initial assessment of *ALOX5* expression pre and post imatinib treatment in human CML demonstrated that *ALOX5* was down-regulated below normal, unlike the mouse model of CML which showed that *ALOX5* was up-regulated. Following imatinib treatment, *ALOX5* mRNA expression increased in the CCRe and No-CCRe groups. In patients who subsequently progressed to BC, no change in *ALOX5* expression was observed. The failure to increase *ALOX5* expression following 3 months of imatinib treatment may identify those patients at risk of disease progression.

ALOX5 function was assessed by measuring plasma LTB4. LTB4 was found to increase in all three groups of patients following imatinib treatment. LTB4 was also found to be increased in BC compared to CP. These data suggest that the arachidonic acid pathway (Figure 6.5) is functionally active to the point of LTB4 production.

To determine the differences between the *ALOX5* pathway observed in mice and the clinical samples studied, the LTB4 receptor BLT1 was investigated. LTB4 acts by binding to BLT1 in order to mediate its positive role on *ALOX5*. At diagnosis of CML patients had very low levels of BLT1 protein compared to normal samples. Following imatinib treatment BLT1

levels increased towards normal, and in the one BC case studied BLT1 protein was further suppressed. These findings help to explain the data seen in CML patients. At diagnosis of CML, *ALOX5* gene expression was found to be suppressed and there was an accumulation of LTB4. The accumulation of LTB4 is likely due to lack of the LTB4 receptor BLT1. A lack of BLT1 allows the intermediates of the pathway namely 5-HEPTE and LTA4 to suppress *ALOX5* gene expression, and thus LTB4 accumulates as it has no receptor to bind to. Following imatinib treatment BLT1 protein increases; thus LTB4 can bind to its receptor and positively regulate *ALOX5* as demonstrated by an increase in ALOX5 protein and gene expression.

In patients with atherosclerosis the binding of LTB4 to BLT1 induces the rapid phosphorylation of mitogen activated protein kinases (MAPK, ERK1/2 and JNK1/2), and PI3K/AKT, and also increases NF-κB activation,[245] which are all targets of BCR-ABL1. It is therefore interesting to speculate that CML cells attempt to switch off excessive signalling via these pathways by down-regulating BLT1 in order to try and maintain cellular homeostasis. This hypothesis warrants further investigation but is outside the scope of this thesis.

In conclusion, it is apparent that two different pathways exist with regards to *ALOX5* between the CML mouse model and CML patients. This accounts for the difference between the clinical samples studied herein and the results of the Chen *et al*[253] paper. Early in this study, it became clear that the pursuit *ALOX5* as a therapeutic target in CML using the *ALOX5* inhibitor zileuton was not feasible, and this aspect of the study was abandoned.

CHAPTER SEVEN - The role of PP2A and its inhibitor SET in CML patients

7.1.0 INTRODUCTION

The majority of CML patients present in a chronic phase, which is nowadays readily controlled by imatinib and related agents. However, at least one-third of CML patients treated with imatinib will eventually fail treatment,[21,287] and a significant proportion of these will progress towards blast crisis. Poor response to imatinib and progression to blast crisis have been linked to high BCR-ABL1 tyrosine kinase activity,[303] but why one patient can remain in well controlled chronic phase for decades, whereas another rapidly progresses to blast crisis is poorly understood.

Many cells utilise reversible phosphorylation as a mechanism of post-translational modification for activating and deactivating key regulatory molecules involved in cell signalling.[254] A major cellular serine/threonine phosphatase working in opposition to kinases is the Protein Phosphatase 2A (PP2A).[254] PP2A plays an important role in regulating cell proliferation, differentiation and survival; loss of function has been associated with cellular transformation; thus it is also known as a tumour suppressor (as discussed in section 1.8.0).[256] This chapter investigates the predictive value of assessing the tumour suppressor PP2A and its inhibitory protein SET (and its binding protein SETBP1) in newly diagnosed CML patients at diagnosis and following imatinib treatment.

7.2.0 PATIENT COHORT

Patients were only included in this study if they had received imatinib 400mg from initial diagnosis. Additionally, patients also needed to have paired MNC and cDNA samples, at both diagnosis and at 12 months of treatment, or at disease progression. 31 patients fulfilled these criteria; their characteristics are described in Table 7.1.

Table 7.1. Patient clinical characteristics.

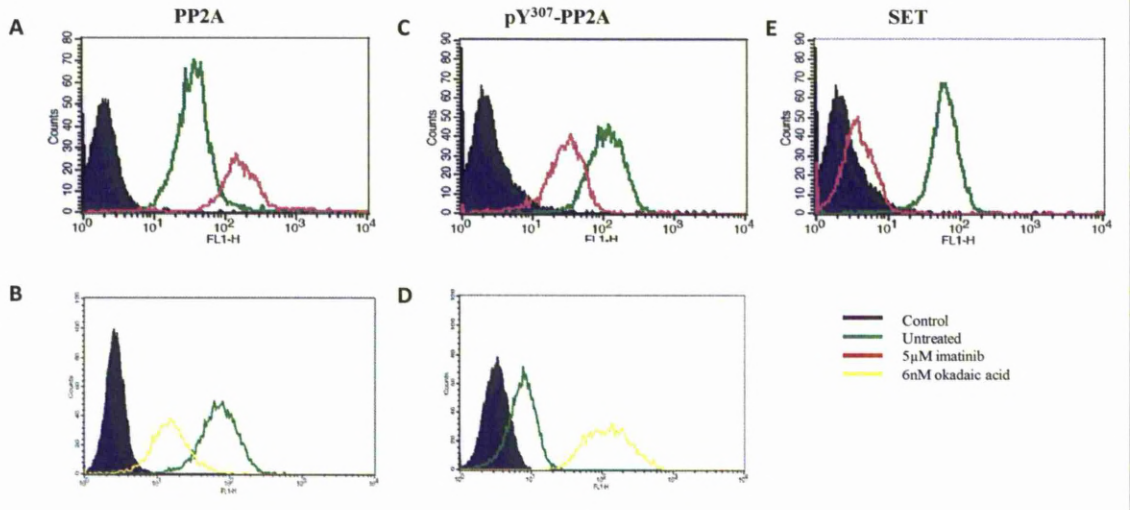
				Sokal score			
	No. patients	**M/F**	**Mean age (range)**	**Low**	**Intermediate**	**High**	**Unknown**
CCRe	14	7/7	45 (19-60)	6	1	4	3
No CCRe	11	7/4	48 (23-67)	2	2	5	2
BC	6	5/1	37 (24-59)	1	2	2	1
Total	31	19/12	44 (19-67)	9	5	11	6

7.3.0 RESULTS

7.3.1 Optimisation of FACS methodology for the detection of PP2A, pY^{307}-PP2A and SET.

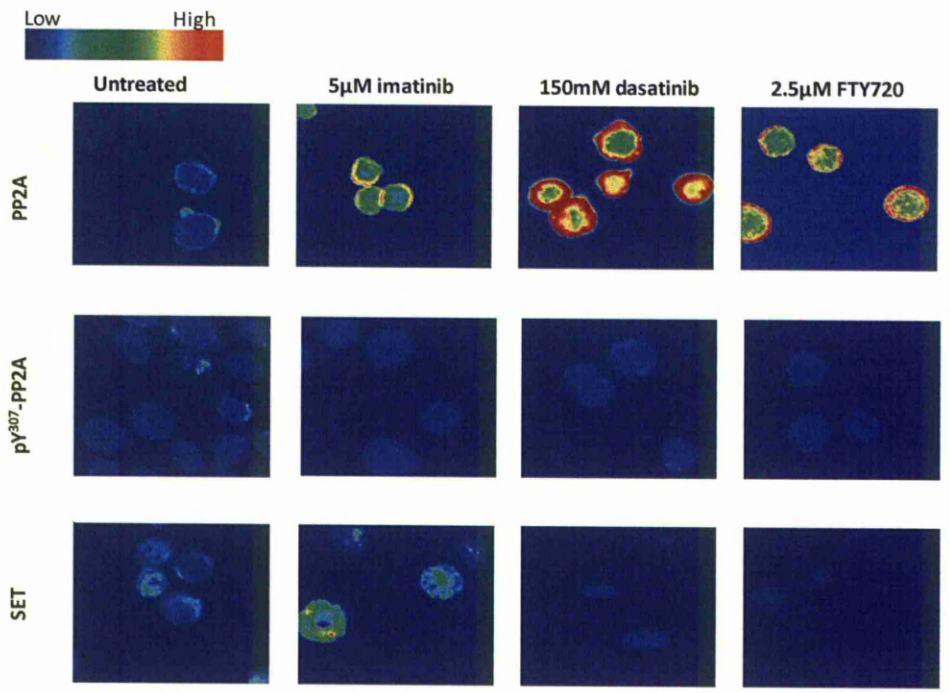
Fixation and permeabilisation optimisation for CML cells has been addressed in chapter four. All antibody concentrations were optimised to ensure maximal signal intensity (data not shown). To validate the PP2A detection methodology, K562 cells were treated with imatinib to reactivate PP2A^{264} and okadaic acid, a known inhibitor of PP2A. Imatinib treatment resulted in an increase in PP2A as demonstrated by a peak shift to the right, and conversely okadaic acid inhibited PP2A as demonstrated by a peak shift to the left (Figures 7.1A and B respectively). PP2A is inactive when phosphorylated at tyrosine 307 (pY^{307}-PP2A). Imatinib reduced and conversely okadaic acid increased PP2A phosphorylation, further inactivating PP2A (Figures 7.1C and D). Reactivation of PP2A following imatinib treatment also coincided with a decrease in the inhibitor SET (Figure 7.3E).

Figure 7.1. Optimisation of PP2A, pY^{307}-PP2A and SET FACS assays.
K562 cells treated with 5μM imatinib and 6nM okadaic acid (inhibitor of PP2A) for 24 hours



Reactivation of PP2A was investigated further using imatinib, dasatinib and the PP2A activator FTY720. Confocal analysis of K562 cells treated with imatinib showed an increase in PP2A accompanied by a decrease in pY^{307}-PP2A. Imatinib resulted in an increase in SET; although at first sight this is surprising, this will be discussed later in relation to No-CCRe patient samples (section 7.3.7). Dasatinib treatment increased PP2A protein, and this increase was superior to that observed with imatinib. Both pY^{307}-PP2A and SET also decreased significantly, compared to the untreated control. The results of FTY720 treatment were similar to those observed with dasatinib (Figure 7.2).

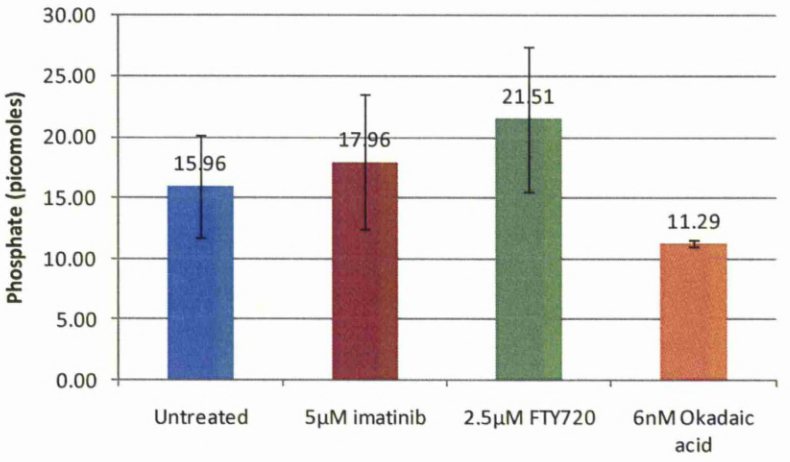
Figure 7.2. Changes in PP2A, pY^{307}-PP2A and SET following imatinib, dasatinib and FTY720 treatment in K562 cells.
Following 24 hours of imatinib treatment PP2A protein increases and the amount of pY^{307}-PP2A decreases but SET increases. Dasatinib and FTY720 treatment resulted in an increase in PP2A protein and a decrease in both pY^{307}-PP2A and SET leading to the reactivation of PP2A.



7.3.2 PP2A activity

PP2A activity was assessed using K562 cells treated with imatinib, FTY720 and okadaic acid for 24 hours using a methodology kindly supplied by Professor Danilo Perrotti, Ohio State University, Columbus, Ohio, USA (Figure 7.3). Untreated K562 cells had a baseline phosphatase activity of 15.96 pico-moles, and following imatinib and FTY720 treatment this increased slightly by 2 and 5.5 pico-moles respectively. As expected okadaic acid resulted in a decrease in PP2A activity to 4.67 pico-moles. Although these treatments altered the PP2A activity as anticipated, the changes were small (2-5 pico-moles) and were not statistically significant.

Figure 7.3. PP2A activity in K562 cells.
Following 24 hours of imatinib, FTY720 and okadaic acid treatment PP2A activity was assessed. Imatinib and FTY720 increased PP2A activity while okadaic acid decreased PP2A activity.



Detection of PP2A activity is extremely difficult to determine. Prior to adopting the methodology of Professor Perrotti, a commercial kit was used unsuccessfully and given the insignificant changes observed in the CML cell line, it was decided not to extend this assay to precious primary clinical material. Other groups working on PP2A have also experienced problems reliably detecting PP2A activity in primary material.[264,265,319] They have used PP2A phosphorylation status instead, since PP2A is inactive when phosphorylated at tyrosine 307.

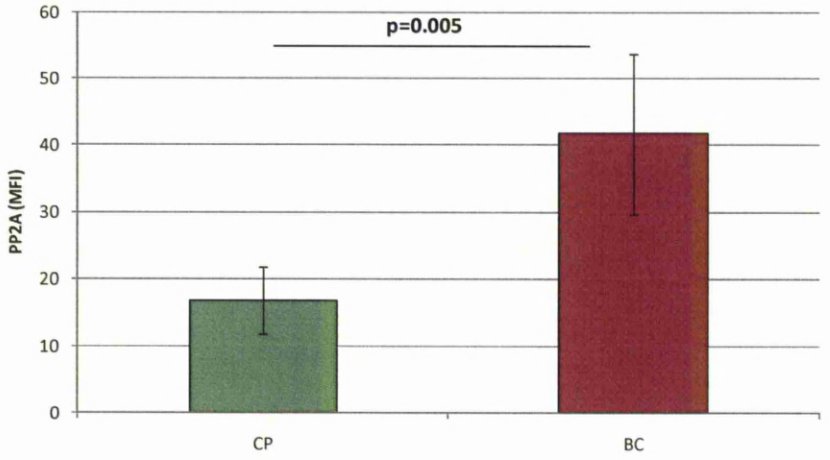
7.3.3 Determining the normal level of PP2A, pY^{307}-PP2A and SET.

Before investigating the level of PP2A, pY^{307}-PP2A and SET in CML samples it was important to determine the normal level of each protein. To do this, MNC from 10 healthy volunteers were collected and each protein was assessed by FACS. The normal levels were determined as: PP2A 21.4 MFI (range 17.1-25.7), pY^{307}-PP2A 1.6 (range 0.8-2.3) and SET 0.2 MFI (range 0.1-0.3).

7.3.4 PP2A protein is elevated during blast crisis.

Previous work by others has implicated PP2A function in the pathophysiology of CML and AML.[264,265] To further investigate the importance of PP2A protein expression, PP2A levels in the MNC from CML patients in chronic phase and blast crisis were compared (Figure 7.4.). PP2A protein was found to be significantly elevated in the cells from patients in blast crisis ($p=0.05$). These data suggest a potential importance for PP2A. In view of PP2A's role as a tumour suppressor, these results were not anticipated.

Figure 7.4. Level of PP2A protein at chronic phase and blast crisis.
PP2A protein is higher in blast crisis samples compared to chronic phase samples.



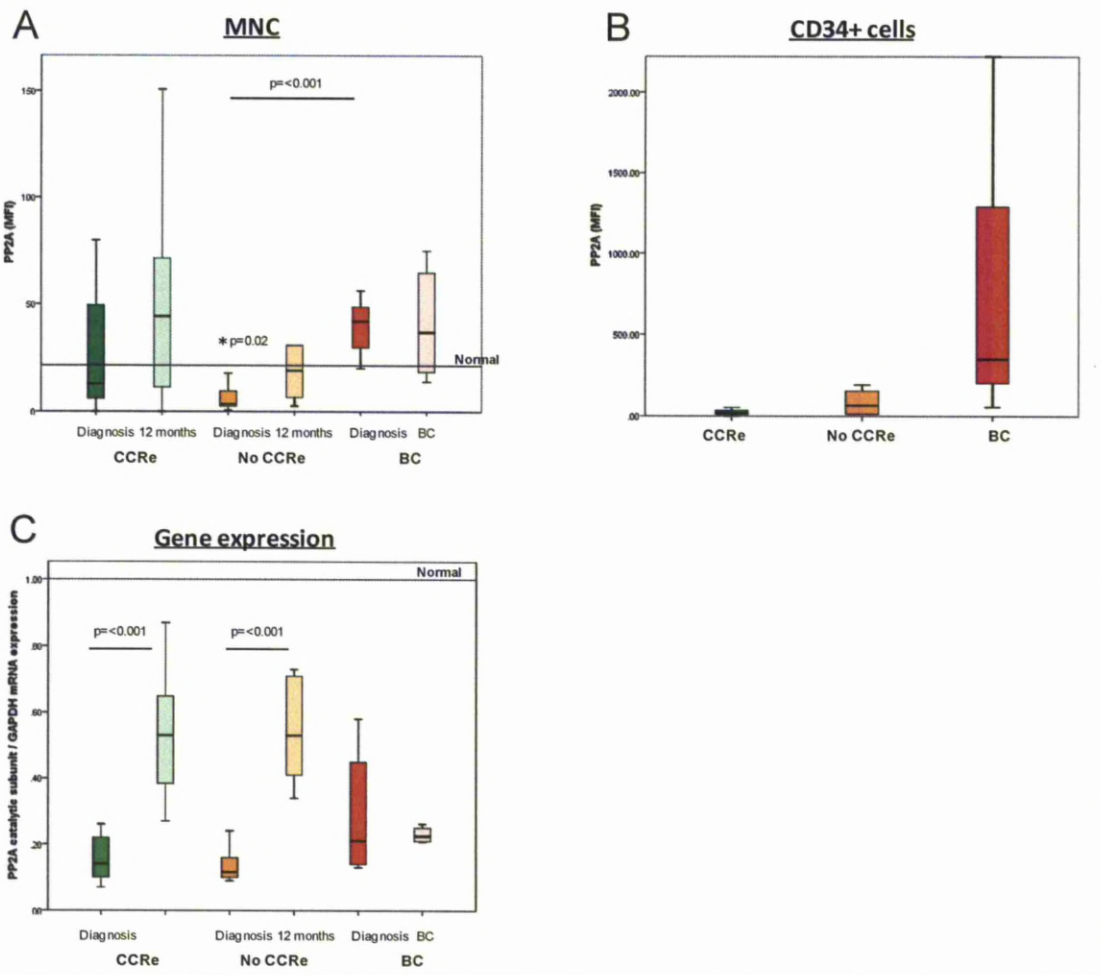
7.3.5 PP2A protein expression is increased in MNC and CD34+ cells from patients destined to progress to blast crisis.

In order to determine whether PP2A protein levels were indicative of disease progression, PP2A protein was analysed in MNC of 31 patients who were in chronic phase at diagnosis and again in the same patients after 12 months of treatment or at transformation. The clinical response of these patients was stratified into three groups depending on outcome; CCRe, No-CCRe and BC. No BCR-ABL1 kinase domain mutations were detected in any patients. At diagnosis the mean PP2A protein level in patients who later achieve CCRe was not significantly different from that in normal MNC, whereas that in patients destined not to achieve CCRe (No-CCRe) was significantly lower than in normal cells ($p=0.02$) (Figure 7.5). In both groups of patients PP2A levels increase following 12 months of treatment. In contrast to the CCRe and No-CCRe patients, PP2A levels in patients destined to progress into blast crisis were much higher ($p=<0.001$ compared to the No-CCRe group), and these levels did not change following treatment (Figure 7.5A). When the same experiment was performed on

CD34+ cells from the same patients, similar results were obtained (Figure 7.5B); PP2A protein was elevated in the diagnostic CD34+ cells from patients who subsequently progress into BC compared to the CCRe and No-CCRe groups. These data suggest that PP2A is not only elevated at BC but also at diagnosis in patients destined to progress to blast crisis. Furthermore, increased PP2A protein expression in MNC and CD34+ cells at diagnosis may indicate a higher probability of disease progression.

To determine whether PP2A protein levels were a function of PP2A gene expression, qRT-PCR was used to measure expression of the gene coding for the PP2A catalytic subunit. The pattern of PP2A gene expression was similar to that of PP2A protein expression in the CCRe and No-CCRe groups. PP2A mRNA levels were lower at diagnosis and increased significantly following 12 months of treatment ($p=<0.001$), whereas in the BC group diagnostic PP2A mRNA levels were higher than in the CCRe and No-CCRe groups but did not change during treatment (Figure 7.5C). Interestingly, PP2A gene expression in all three groups, regardless of whether the sample was taken at diagnosis or after treatment, was lower than that observed in normal cells. Since protein levels of PP2A in the MNC from the BC group were higher than in normal MNC, this suggests a role for post-translational modification in the stabilisation of PP2A protein expression in the BC group.

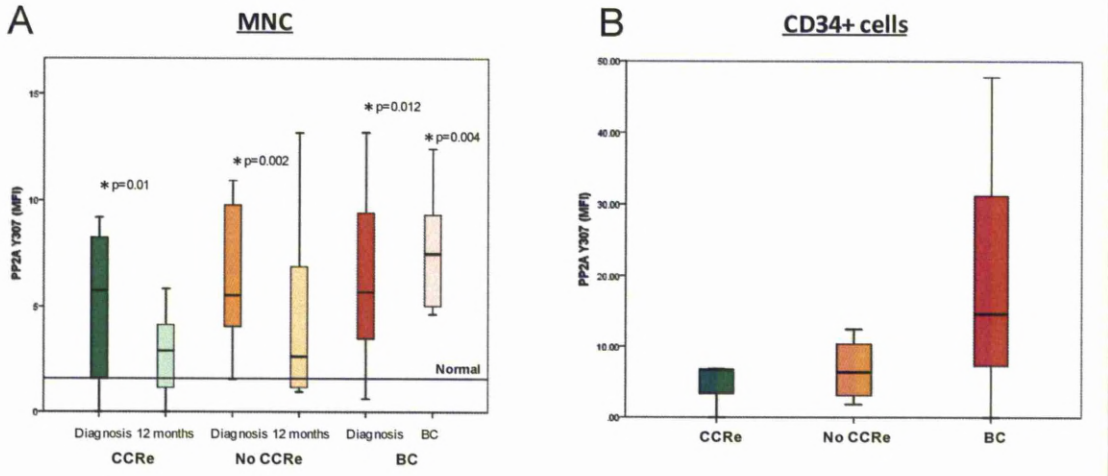
Figure 7.5. PP2A protein expression is increased in MNC and CD34+ cells from patients destined to progress to blast crisis.
The horizontal line represents the mean normal level observed in ten healthy volunteers. **Panel A:** Levels of PP2A protein as assessed by flow cytometry. The level of PP2A is high in patients destined to progress to blast crisis, in comparison to No-CCRe patients (p=<0.001). * denotes a statistically significant difference between No-CCRe and normal (p=0.002).
Panel B: Level of PP2A protein in diagnostic CD34+ cells. The level of PP2A is high in patients destined to progress to blast crisis.
Panel C: Expression of the PP2A catalytic subunit, as assessed by qRT-PCR. Expression was lower than in normal MNC for all three response groups at both diagnosis and at 12 month/transformation follow up. Expression significantly increased following imatinib treatment in both the CCRe and No-CCRe groups (p=<0.001) but no change was observed in the BC group.



7.3.6 pY^{307}-PP2A status is increased in MNC and CD34+ cells from patients destined to progress to blast crisis.

PP2A is inactive when phosphorylated at tyrosine 307 (pY^{307}-PP2A), and this can be used to indicate its activity level.[264,265,319] Phosphorylated levels of PP2A were significantly greater at diagnosis in the CCRe ($p=0.01$) and No-CCRe ($p=0.002$) groups, and in patients who progressed into BC ($p=0.012$) and at BC ($p=0.004$), compared to normal MNC (Figure 7.6A). Next, the PP2A phosphorylation status was assessed in CD34+ cells from patients in these response groups. pY^{307}-PP2A was elevated in cells from the CCRe and No-CCRe response groups, but dramatically increased levels were found in cells from patients destined to progress (Figure 7.9B). Thus, although Figure 7.5A demonstrated high PP2A protein levels in patients destined to progress, this was found to be functionally inactive (phosphorylated). These data support the conclusions of a previous report indicating a role for PP2A inactivation in the pathology of CML.[264] Taken together, these data demonstrate that PP2A is functionally inactive in the MNC and CD34+ cells of patients destined to progress to blast crisis.

Figure 7.6. pY^{307}-PP2A at diagnosis and following 12 months of treatment or at progression, stratified by response.
Panel A: Level of pY^{307}-PP2A (inactive), as assessed by flow cytometry. The mean level in 10 healthy subjects = 1.6 (range 0.8-2.3). Phosphorylated levels of PP2A were greater at diagnosis in CCRe (p=0.01) and No-CCRe (p=0.002) groups, and in patients who progressed into BC (p=0.012) and at BC (p=0.004), compared to normal MNC. **Panel B:** Level of pY^{307}-PP2A (inactive) in CD34+ cells. The degree of phosphorylation is greater patients destined to progress into blast crisis.



7.3.7 SET and *SET binding protein 1 (SETBP1)* levels are not indicative of clinical outcome.

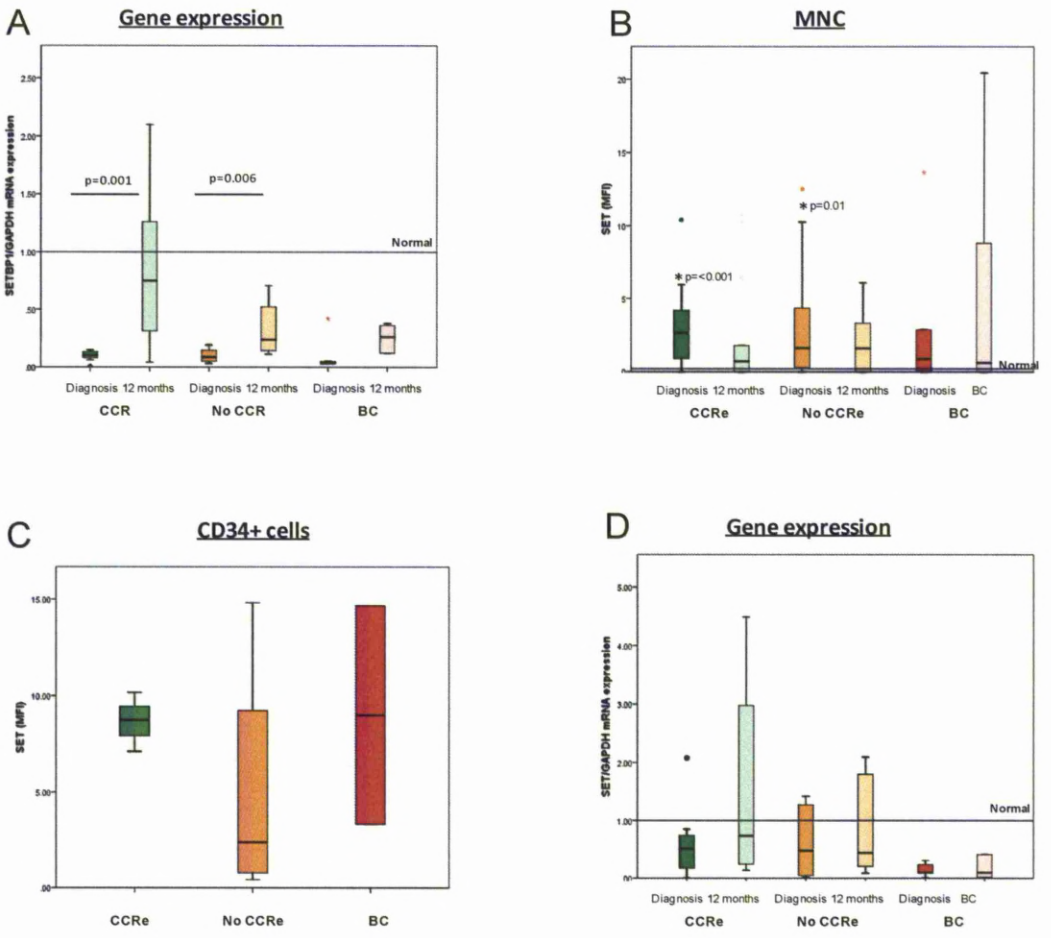
SET and SETBP1 have respectively been implicated in regulating PP2A activity in the leukaemic cells of CML and AML.[264,265] *SET* and *SETBP1* expression were assessed at diagnosis and again following 12 months of treatment or at transformation. No statistically significant difference in *SETBP1* expression was observed between the CCRe, No-CCRe and BC clinical groupings of CML (Figure 7.7A). Interestingly, *SETBP1* expression increased significantly following 12 months treatment in the CCRe and No-CCRe groups but not in the BC group (p=0.001 and p=0.006 respectively). Moreover, and unlike in the leukaemic cells

of AML,[265] *SETBP1* gene expression levels were lower in CML samples when compared to those from healthy volunteers.

SET protein levels (Figure 7.7B) were significantly higher at diagnosis in the CCRe and No-CCRe groups compared to normal (p=<0.001 and p=0.01 respectively). In patients who subsequently progressed to BC there was a trend for lower SET levels. This trend was also seen for SET mRNA levels (Figure 7.7D), since samples from patients who later progressed had lower levels of SET mRNA than did samples from either CCRe or No-CCRe patients. Interestingly, in BC samples at transformation, SET protein levels increased to become spread over a higher range than at diagnosis, and may suggest that SET plays a role during disease progression. This observation contrasts with the fall in SET expression observed in samples from patients within the CCRe and No-CCRe groups following 12 months of treatment. SET protein expression was also assessed in diagnostic CD34+ cells and no apparent difference between the three clinical groups was observed (Figure 7.7C).

Taken together, these results are broadly in agreement with previously reported data,[264] but also suggest that SET may not be the only factor inhibiting PP2A in the malignant cell, especially in the case of patients destined to progress to blast crisis. This implies that another mechanism may be inhibiting PP2A in this group of patients.

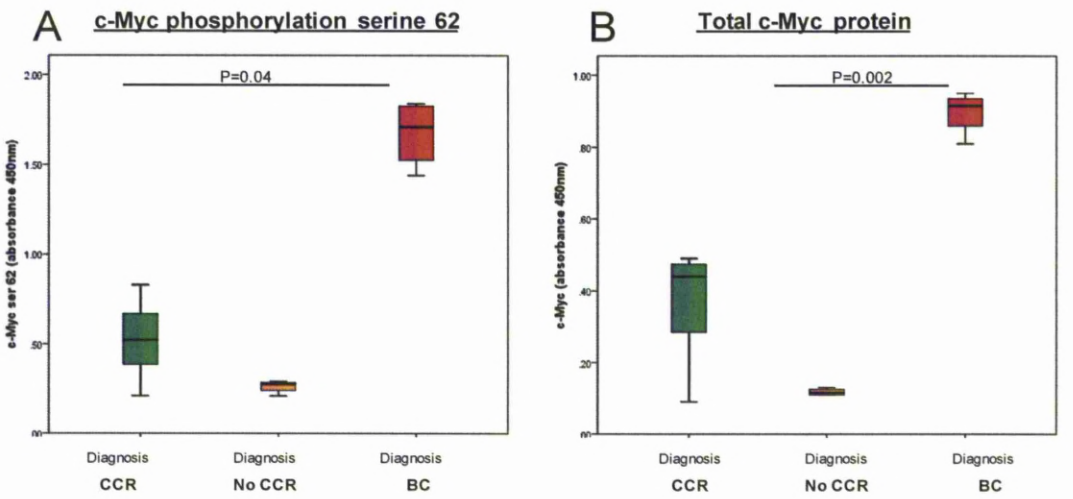
Figure 7.7. SET and SETBP1 at diagnosis and follow-up.
SET protein is low at diagnosis in patients who subsequently progress to blast crisis. **Panel A:** mRNA expression of *SET binding protein (SETBP1).* No significant differences in *SETBP1* expression were observed between the three response groups. Following 12 months of imatinib treatment *SETBP1* expression increases in the CCRe and No-CCRe groups (p=0.001 and 0.006 respectively). **Panel B:** Levels of SET protein. SET protein levels are higher than in normal MNC in the CCRe and No-CCRe groups (p=<0.001 and p=0.01 respectively). **Panel C:** Levels of SET protein in CD34+ cells. No difference observed. **Panel D:** SET mRNA expression.



7.3.8 Inactive PP2A functions to stabilises c-Myc

Under normal conditions PP2A functions to dephosphorylate kinases including c-Myc. c-Myc cannot be degraded when phosphorylated at serine 62, and remains oncogenic.[271] To test whether pS^{62}c-Myc is a factor in CML pathophysiology, an ELISA assay was used to measure the levels of pS^{62}-Myc in diagnostic samples. MNC from patients destined to progress into BC had significantly higher levels of pS^{62}c-Myc than did those from patients within either the CCRe or No-CCRe groups (Figure 7.8A, p=0.04). Since S^{62} phosphorylation of c-Myc stabilises this protein against degradation,[271] the total c-Myc protein level was also measured in the same samples, to determine if increased stabilisation also gave rise to increased protein levels, and similar results were observed. c-Myc protein levels were significantly higher in the cells of patients who subsequently progress to blast crisis (Figure 7.8B, p=0.002). These data strongly suggest that high levels of c-Myc and phosphorylation at serine 62 indicate a high risk of disease progression. Since PP2A is the major phosphatase that dephosphorylates c-Myc, these results further suggest that PP2A function is inhibited at diagnosis.

Figure 7.8. Assessing PP2A function - c-Myc is elevated at diagnosis in patients destined to progress into blast crisis.
Panel A: Levels of pS^{62}c-Myc. **Panel B**: Total c-Myc protein levels, stratified by clinical outcome. CCRe n=10, No-CCRe n=5 and BC group n=5.



7.4.0 DISCUSSION

The data presented in this chapter demonstrate that at diagnosis patients destined to achieve a response to imatinib treatment (CCRe group) had low levels of PP2A protein, low PP2A activity (as indicated by high levels of pY^{307}-PP2A) and high levels of SET protein, indicating impaired PP2A function controlled by the PP2A inhibitor SET. Following 12 months of imatinib treatment the levels of PP2A increased, pY^{307}-PP2A decreased and the levels of SET decreased – all leading to an environment permitting achievement of CCRe. This is consistent with previously reported cell line data[264] which demonstrated that PP2A is inhibited by SET, and that function can be restored by imatinib treatment *in vitro*. The present data are additive to the current literature, confirming the cell line findings of Neviani *et al*[264] in our clinical material.

In the No-CCRe group the level of PP2A was significantly lower than that observed in normal blood and its activity was low (high pY^{307}-PP2A). SET protein levels were also high thus inhibiting PP2A. Following imatinib treatment PP2A levels increased and the amount of pY^{307}-PP2A decreased, suggesting that the activity of PP2A had been restored. However, these changes were not accompanied by an alteration in levels of the inhibitory protein SET suggesting that PP2A function may be only partially restored compared to diagnosis. This may contribute to why this group of patients have not achieved a CCRe.

In sharp contrast, patients at diagnosis who were destined to later progress into blast crisis were found to have significantly higher levels of PP2A both at diagnosis and at blast crisis. The degree of pY^{307}-PP2A was higher (low activity) than normals. Interestingly at diagnosis SET levels were virtually undetectable, although SET did increase during transformation, suggesting that SET does not play a role in inhibiting PP2A function in this particular group

of patients. The PP2A and pY^{307}-PP2A trends observed in MNC were confirmed in CD34+ cells.

Given that PP2A function is not inhibited by SET in those patients destined to progress to blast crisis, an alternative mechanisms of PP2A inhibition was investigated, namely *SETBP1*. Overexpression of *SETBP1*, the binding protein for SET, predicts adverse outcome in elderly patients with acute myeloid leukaemia (AML).[265] Although SET has been shown to play a role in inhibiting PP2A in the CCRe group, the data presented here suggest that, in contrast to AML, *SETBP1* is not a diagnostic prognostic marker of clinical outcome following imatinib treatment in CML. Other factors may therefore be of greater importance /influence in the regulation of PP2A in CML.

Patients destined to progress into blast crisis had high levels of PP2A, which did not fit the prediction that PP2A levels would be low in CML samples. Patients destined to progress have high PP2A that is not functionally inhibited by either SET or *SETBP1*. It was therefore necessary to confirm that PP2A function was indeed impaired. pS^{62}c-Myc was used as a functional readout of PP2A activity. Under normal conditions PP2A dephosphorylates c-Myc and thus targets c-Myc for degradation. In this chapter I have demonstrated that pS^{62}c-Myc and total c-Myc protein are high in patients destined to progress, confirming that PP2A is functionally inactive. It is known that c-Myc may contribute to disease progression in CML, mediated through aneuploidy.[96,97,320,321] c-Myc has a critical role in enhancing cell proliferation and it is therefore possible that this results in an increase in DNA mismatch repair errors leading to an increase in genomic instability.[96]

In conclusion, it is apparent that PP2A is functionally inactivated at diagnosis in patients destined to subsequently progress to BC; however, the mechanism responsible for this remains unclear. This will be addressed in the following chapter.

CHAPTER EIGHT - Cancerous inhibitor of PP2A (CIP2A) at diagnosis of chronic myeloid leukaemia is a critical determinant of disease progression

8.1.0 INTRODUCTION

In the previous chapter it was apparent that PP2A is functionally inactivated at diagnosis. In patients destined to achieve a CCRe, PP2A is inhibited by SET. However, the mechanism of PP2A inactivation in patients destined to progress remains unclear and does not appear to be due to SET. The aim of this chapter is to further dissect the PP2A signalling pathway, to understand how PP2A is inactivated in patients destined to progress into blast crisis.

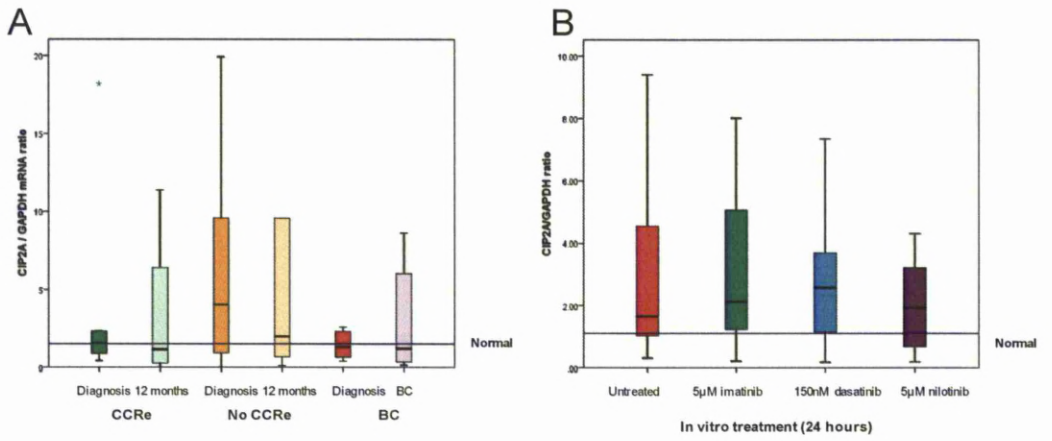
CIP2A was first described in 2007 as an alternative inhibitor of PP2A.[271] CIP2A has since been shown to be an adverse prognostic feature in both breast[93] and gastric[272] cancer. The role of CIP2A is detailed more fully in section 1.8.6. This chapter investigates the role of CIP2A in CML patients at diagnosis and following imatinib treatment.

8.2.0. RESULTS

8.2.1. CIP2A mRNA expression does not correlate with clinical outcome.

The patient cohort previously described in section 7.2.0 was used in this study. Figure 8.1A shows CIP2A gene expression as assessed by qRT-PCR at diagnosis and following 12 months of imatinib treatment or at disease progression. No statistically significant difference was observed between any of the three response groups or at either time point, nor were any of these different from normal MNC. Given the previous report in both breast and gastric cancers that CIP2A mRNA expression correlated with clinical outcome,[91,93] this is not the case in the CML samples studies. Using an *in vitro* assay, MNC from 18 newly diagnosed CML patients were cultured with either 5μM imatinib, 150nM dasatinib or 5μM nilotinib for 24 hours and changes in CIP2A mRNA expression were measured (Figure 8.1B). Following TKI treatment no change in CIP2A mRNA expression was observed.

Figure 8.1. CIP2A expression levels at diagnosis and follow-up.
Panel A: mRNA expression of CIP2A. No statistically significant difference was observed between any of the three response groups. **Panel B:** Changes in CIP2A mRNA expression in newly diagnosed CML patients following *in vitro* treatment with TKI. No statistically significant difference was observed (n=18).



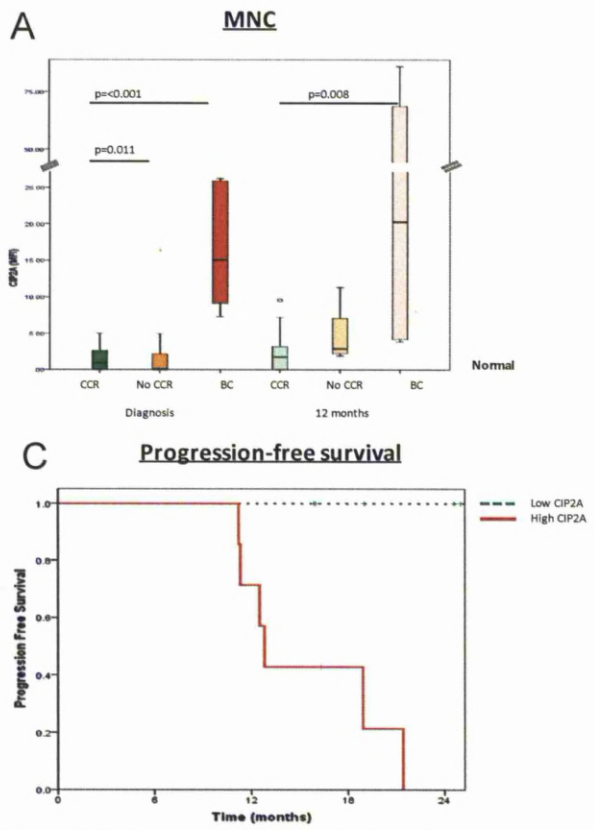
8.2.2 Cancerous inhibitor of PP2A (CIP2A) protein levels predict blast crisis.

In sharp contrast, Figure 8.2A demonstrates that CIP2A protein levels in MNC taken at diagnosis were significantly higher in patients who later progressed to blast crisis than in patients from the CCRe and No-CCRe groups ($p=<0.0001$ and $p=0.01$ respectively). This observation was also confirmed in CD34+ cells; samples from patients destined to progress into BC had much higher levels of CIP2A than either CCRe or No-CCRe patients ($p=0.017$, Figure 8.2B). No correlation was seen between CIP2A expression and Sokal score (data not shown). These results suggest that CIP2A may be important in inhibiting PP2A function in CML patients who progress to blast crisis. The importance of increased CIP2A protein expression in disease progression is further exemplified following 12 months of treatment; whereas CIP2A protein levels remain essentially unchanged in samples from the CCRe and No-CCRe groups, the level increases further at disease progression ($p=0.008$, Figure 8.2A).

One patient in the No-CCRe had uncharacteristically high CIP2A protein levels (outlier in Figure 8.2A), although this patient did not progress into blast crisis. There are a number of factors to consider in this case. Firstly, unlike the other patients in this study this patient received interferon as part of the SPIRIT 1 clinical trial which might have modified his treatment response from that with imatinib alone.[322] Secondly, this patient switched to a second generation TKI as part of his treatment, thus possibly preventing disease progression and finally, this patient is diabetic and received metformin as part of his treatment. Metformin is also transported by hOCT1 and may therefore have competed with imatinib for access into CML cells; nilotinib transport is not hOCT1 dependent.[298] Metformin has also recently been reported to reactivate PP2A.[323]

Figure 8.2. CIP2A protein level in MNC and CD34+ at diagnosis is predictive of blast crisis.

Panel A: Levels of CIP2A protein. CIP2A protein levels at diagnosis were significantly higher at diagnosis in patients who later progressed to blast crisis than in CCRe or No-CCRe patients (p =<0.0001 and 0.01 respectively). As a patient progresses into BC the CIP2A protein level increases further (p=0.008). **Panel B**: Level of CIP2A protein in diagnostic CD34+ cells stratified by the patients' clinical outcome. CIP2A is elevated in CD34+ cells from patients destined to progress into BC. **Panel C**: Kaplan Meier plot of disease progression, stratified by CIP2A level at diagnosis. Patients with a high diagnostic CIP2A protein level (MFI >5) have 100% probability of progressing to blast crisis by 21 months (p=<0.0001).

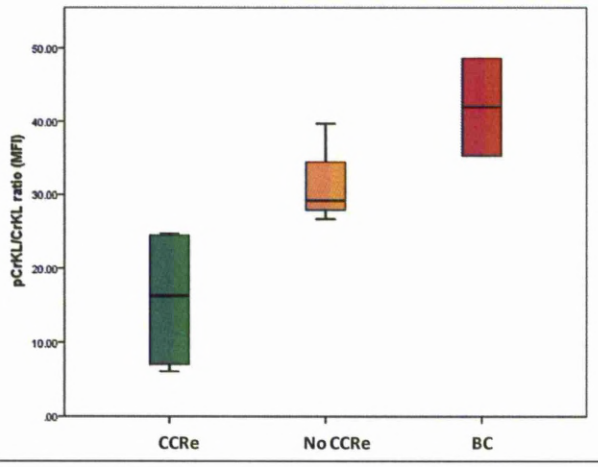


To test whether high expression of CIP2A protein is predictive of disease progression, progression free survival was compared between patients with high (defined as a MFI greater than 5) and low diagnostic CIP2A protein levels. Figure 8.2C shows that the median time to disease progression in patients with high CIP2A protein levels is 13 months, and their probability of progression is 100% at 21 months. Five of these six patients have died of disease progression. In contrast, during the same time period none of the patients with low diagnostic CIP2A levels progressed, and all remain progression-free and alive at their latest follow-up (average follow-up 47 months). These data indicate that CIP2A protein expression is a biomarker of disease progression in CML.

8.2.3 CIP2A protein correlates with high BCR-ABL1 tyrosine kinase activity.

CrkL phosphorylation status is a marker of BCR-ABL1 tyrosine kinase activity.[197,303] Where available, the pCrkL/CrkL ratio was measured at diagnosis using fresh material as previously described (n=10).[303] BCR-ABL1 activity was highest in patients destined to progress into blast crisis (Figure 8.3), demonstrating that high CIP2A protein level and high BCR-ABL1 activity appear to be correlated.

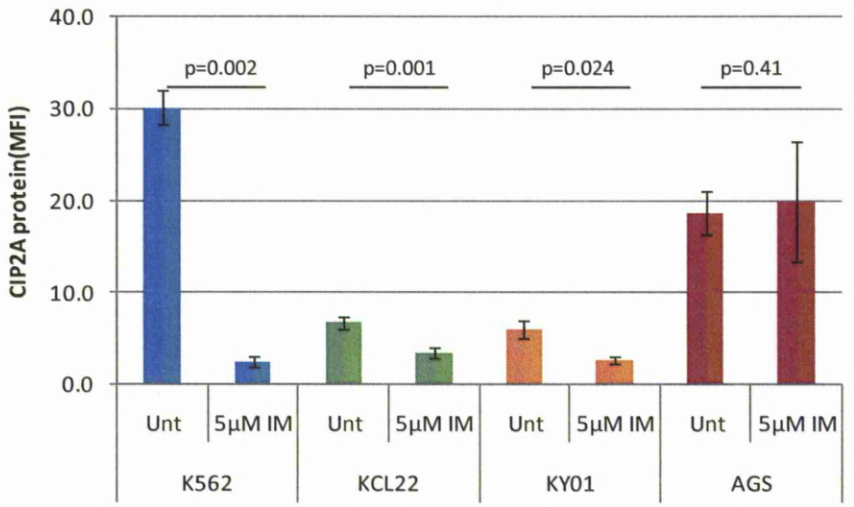
Figure 8.3. BCR-ABL1 tyrosine kinase activity (pCrkL/CrkL ratio) is higher at diagnosis in patients destined to progress into blast crisis.



8.2.4 Is CIP2A protein regulated by BCR-ABL1?

BCR-ABL1 activity is predictive of treatment response in CML.[303] To see whether BCR-ABL1 tyrosine kinase activity was a factor controlling CIP2A protein levels, the BCR-ABL1 positive cells lines K562, KCL22 and KY01 together with the BCR-ABL1-negative gastric cancer cell line AGS (used as a CIP2A positive control)[272] were treated with 5μM imatinib for 24 hours. The results (Figure 8.4) show that imatinib treatment of the BCR-ABL1 positive cell lines resulted in a significant down-regulation of CIP2A protein levels ($p=0.002$ for K562, $p=0.001$ for KCL22 and $p=0.024$ for KY01), but had no effect on CIP2A expression in AGS cells. Thus, BCR-ABL1 activity may be an important factor in the regulation of CIP2A protein expression.

Figure 8.4. Is CIP2A protein regulated by BCR-ABL1?
CIP2A protein levels (mean ± SEM) in CML cell lines K562, KCL22, KY01 and a CIP2A positive gastric cancer cell line AGS, before and after treatment with 5μM imatinib(n=6).



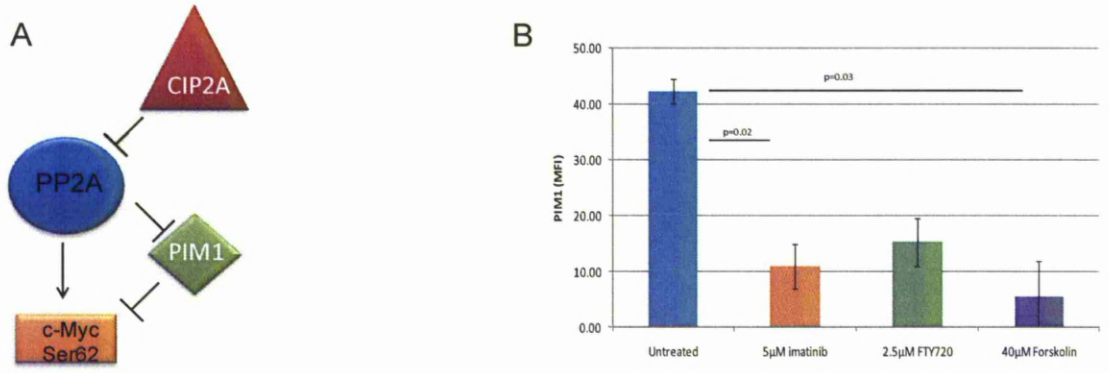
8.2.5 CIP2A inhibits PP2A function and stabilises c-Myc

As discussed in chapter seven c-Myc cannot be degraded when phosphorylated at serine 62, and remains oncogenic (Figure 7.8). Patients who subsequently progress to blast crisis exhibit significantly higher levels of pS^{62}c-Myc as compared to levels seen in the CCRe and No-CCRe groups. Patients in the BC group also had higher total c-Myc protein levels, which was also statistically significant when compared to the No-CCRe group (p=0.002). These data demonstrate that in chronic phase patients who subsequently progress to blast crisis, c-Myc remains phosphorylated at serine 62 thereby stabilising it from degradation. Since PP2A is the major phosphatase dephosphorylating c-Myc, these results further suggest that PP2A function is inhibited by high expression of CIP2A.

8.2.6 PIM1, a surrogate marker for PP2A function and activity.

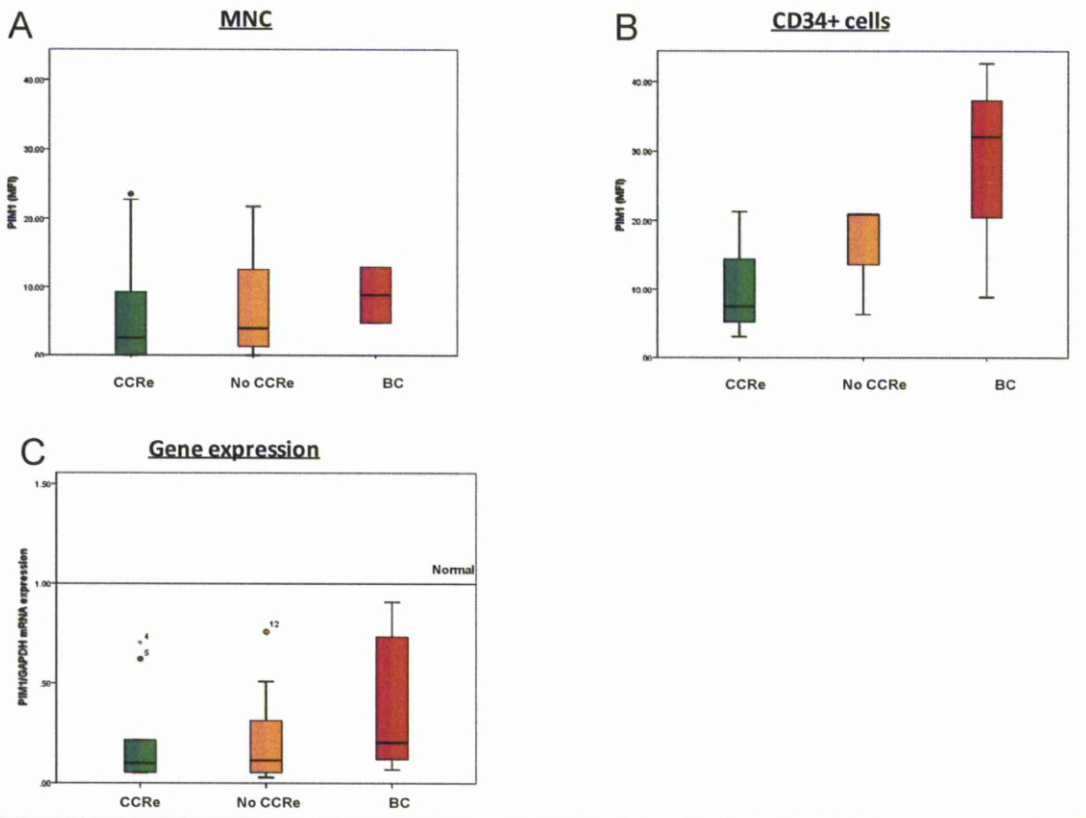
PIM1 is a protein kinase that can phosphorylate and stabilise c-Myc.[92] PIM1 is itself phosphorylated, and in this state it is active and its expression is stable.[274] However, this protein is a target for PP2A, and once it is dephosphorylated by PP2A, it is rapidly degraded in the proteasome (Figure 8.5A).[324] To further confirm that PP2A is functionally inactive in CML, PIM1was investigated as another target of PP2A. Initially K562 cells were treated with 5μM imatinib for 24 hours as this is known to result in the reactivation of PP2A via the suppression of CIP2A (as demonstrated in Figure 8.4). Reactivation of PP2A resulted in a decrease in PIM1 protein (p=0.02, Figure 8.5B). A similar trend was also observed with the PP2A activators FTY720 and forskolin (Figure 8.5B).

Figure 8.5. PIM1
Panel A: Possible mechanisms by which PIM1 could stabilise c-Myc. PIM1 may act as an intermediate step in the stabilisation of c-Myc. **Panel B:** Changes in PIM1 protein level following imatinib, FTY720 and forskolin treatment. K562 cells treated were incubated with imatinib, FTY720 and forskolin for 24 hours (n=4).



In MNC and CD34+ there was a trend for patients destined to progress into BC to have higher levels of PIM1 compared to the CCRe and No-CCRe groups (Figure 8.6A and B). Higher levels of PIM1 protein were observed in CD34+ cells compared to MNC cells. These data are consistent with the view that PP2A is functionally inactive at diagnosis of CML in patients destined to progress into BC. No statistically significant difference was observed in PIM1 mRNA expression (Figure 8.6C). PIM1 expression was below normal in all three clinical groups suggesting that it is regulated post-translationally.

Figure 8.6. PIM1 protein and mRNA expression in MNC and CD34+ cells at diagnosis.
Panel A: Levels of PIM1 protein in MNC. PIM1 is higher at diagnosis in patients destined to progress into BC. **Panel B**: Levels of PIM1 protein in CD34+ cells. PIM1 is higher at diagnosis in patients destined to progress into BC. **Panel C**: PIM1 expression.



8.2.7. CIP2A plays a key role in regulating PP2A and BCR-ABL1 tyrosine kinase activity in CML cells.

To further investigate the effect of CIP2A on CML cells, K562 cells were treated with small interfering RNA (siRNA) targeted at CIP2A (n=5; Figure 8.8). Following siRNA treatment the mean knockdown of the CIP2A protein was 60% (p=0.02; Figure 8.8A). Inhibition of CIP2A resulted in a 63% decrease in BCR-ABL1 tyrosine kinase activity as assessed by the pCrkL/CrkL ratio as previously described[303] (p=0.03; Figure 8.8B). PP2A protein levels remained unchanged (Figure 8.8C), but the degree of PP2A phosphorylation (at Y307, indicating inactivity) decreased by 53%, indicative of an increase in PP2A function (Figure 8.8D). Additionally, SET and JAK2 decreased by 48% and 37% respectively with CIP2A siRNA treatment, indicating that CIP2A acts upstream of JAK2 and SET (Figure 8.8E and F). pS^{62}c-Myc and total c-Myc protein also decreased by approximately 50% (Figure 8.8G and H). Key results from these experiments were also confirmed by Western blotting, using an alternative siRNA sequence, and on the additional CML cell line LAMA84 (Figure 8.9). These data confirm that PP2A is functionally inactivated by CIP2A and that decreasing the level of CIP2A protein removes the block on PP2A function and decreases BCR-ABL1 activity.

Figure 8.8. Effect of CIP2A inhibition by siRNA. Inhibition of CIP2A decreases BCR-ABL1 tyrosine kinase activity.
K562 cells were treated with CIP2A siRNA for 72 hours (n=5). The effects are shown on: CIP2A protein levels as a control (panel A); BCR-ABL1 activity as assessed by the pCrkL/CrkL ratio (panel B); PP2A and phosphorylated PP2A (panels C and D); SET (panel E) and JAK2 (panel F); c-Myc phosphorylation at serine 62 and total c-Myc protein (panel G and H).

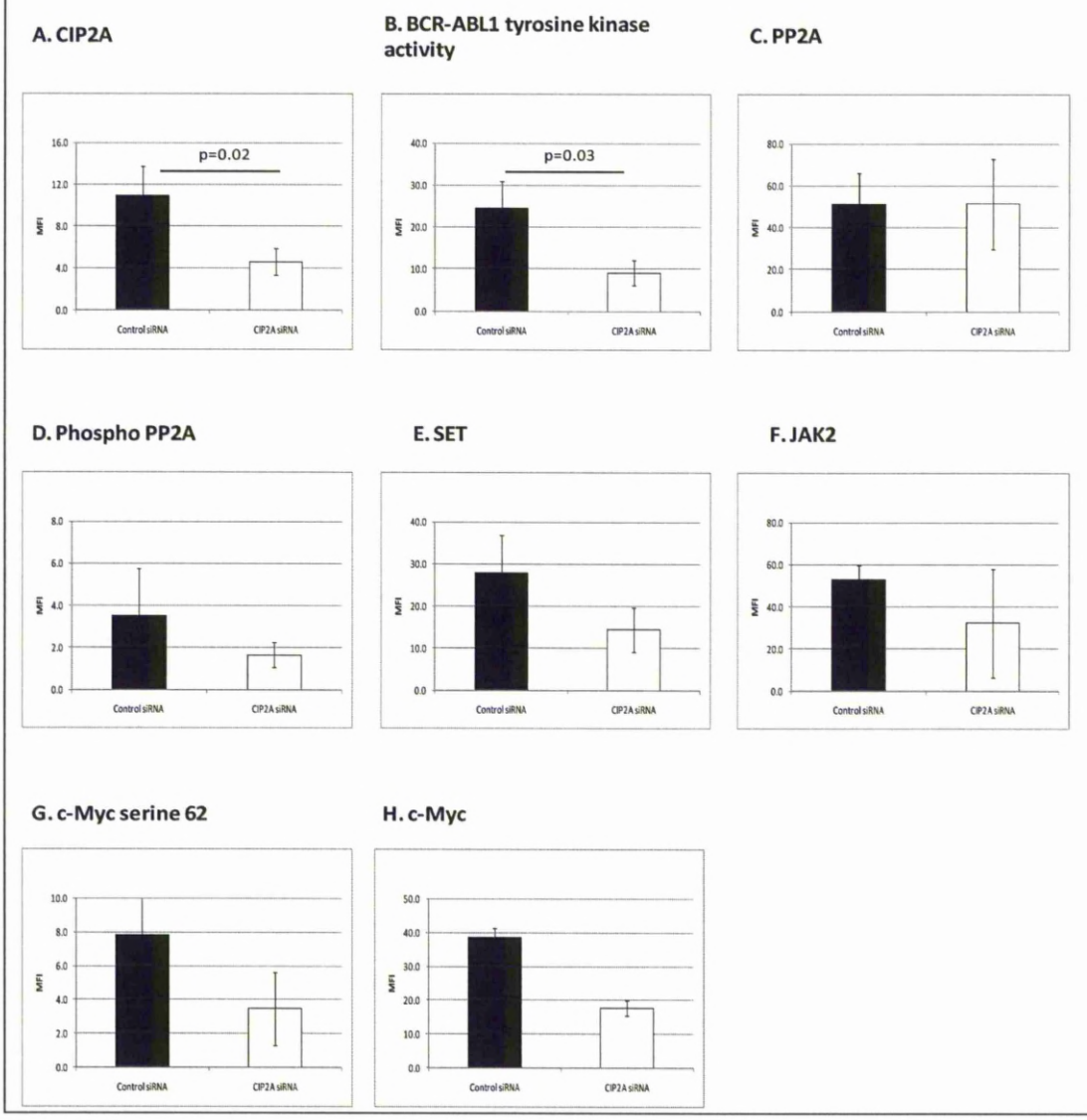
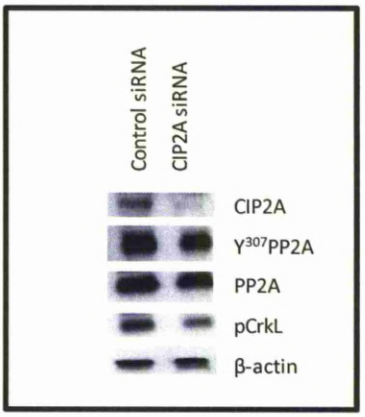


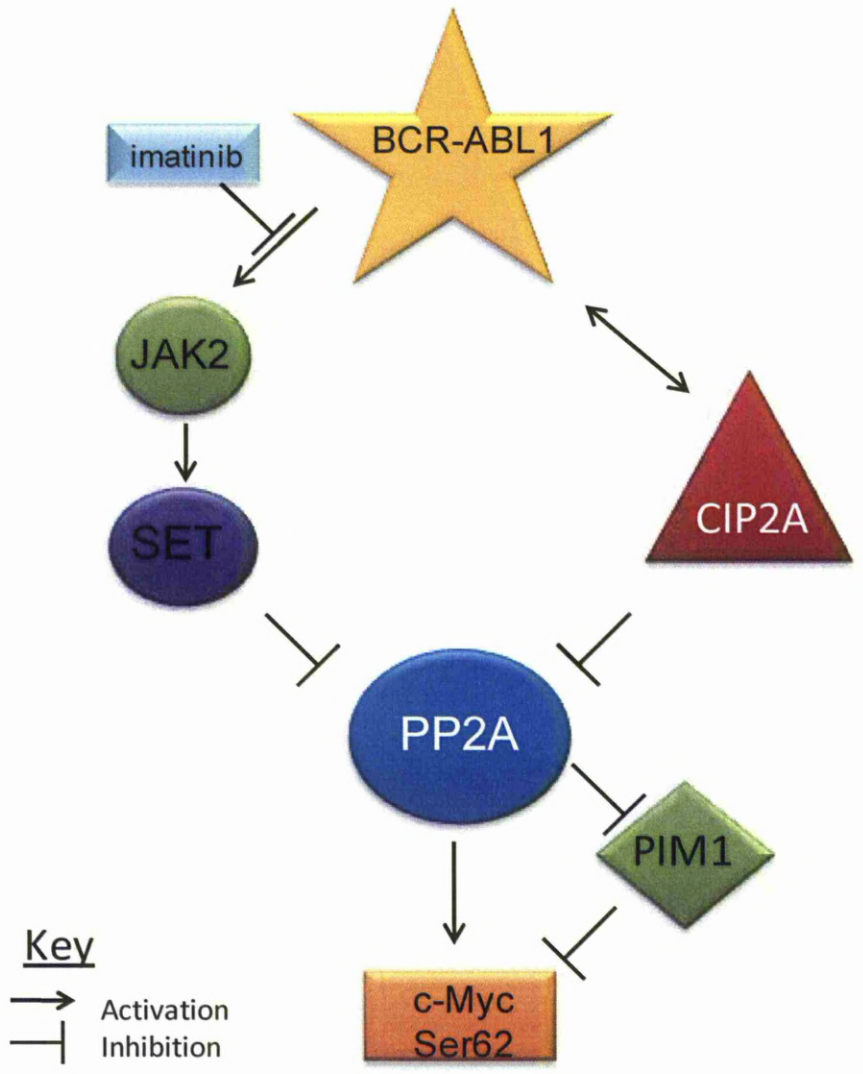
Figure 8.9. CIP2A knockdown in LAMA84 cells – confirms the key siRNA data presented in figure 8.8.
LAMA84 cells were treated with CIP2A siRNA for 72 hours. Key results presented in Figure 8.8 have been confirmed using a different cell line, a different CIP2A siRNA and by a different methodology. CIP2A knockdown in LAMA84 cells reduces Y^{307}PP2A thus reactivating PP2A, and importantly the pCrkL level decreases indicating a decrease in BCR-ABL1 tyrosine kinase activity.



8.2.8 Model mechanisms by which CIP2A regulates PP2A and BCR-ABL1 signalling proteins.

The present data suggest that two different signalling mechanisms may be controlling PP2A in patients with CML. Figure 8.10 summarises the present data and postulates possible mechanisms of how PP2A is inhibited in CML. In most patients, PP2A is suppressed by SET, which is driven by BCR-ABL1 via JAK2. When imatinib treatment is commenced, BCR-ABL1 activity decreases, and leads to removal of PP2A inhibition. This removal of PP2A inhibition permits the dephosphorylation of serine 62 on c-Myc, leading to a decrease in the prevailing c-Myc level. However, in a few patients at diagnosis, CIP2A is present at a high level, and contributes to PP2A suppression. When treatment commences, CIP2A levels may remain high despite a fall in BCR-ABL1 and thus PP2A remains suppressed. c-Myc therefore remains at a higher level.

Figure 8.10. Model mechanisms by which CIP2A regulates PP2A and BCR-ABL1 signalling proteins.
Possible mechanism of PP2A inhibition in CML. In most patients PP2A is suppressed by SET, which is driven by BCR-ABL1 via JAK2. In patients destined to progress into blast crisis PP2A is inhibited by CIP2A.



8.3.0 DISCUSSION

Despite the dramatic effect of imatinib, CML remains a fatal disease for the proportion of patients who progress from chronic phase to blast crisis. Recent data suggest that the second generation TKI nilotinib decreases the rate of progression to BC in the first 12-24 months,[152,325] but it remains to be seen whether this benefit is maintained at later time points. The previous biomarker data on the pCrkL/CrkL ratio (chapter four) and BCR-ABL1 transcript type (chapter five) can only predict patients likely to achieve a CCR, and cannot predict patients destined to progress to blast crisis.[302,303] Various techniques have been used on diagnostic chronic phase samples to predict who will develop blast crisis,[261] and a variety of novel genomic lesions have been identified at blast crisis.[326] It is however not possible at diagnosis to reliably predict which patients will develop disease progression. This is in part due to a poor understanding of the factors facilitating development of the novel genomic lesions that lead to disease progression.

The present data suggest that CIP2A is an important determinant of future disease progression in CML. High CIP2A protein levels are present in diagnostic MNC and CD34+ cells of patients destined to progress into blast crisis, and following imatinib treatment CIP2A levels increase further. The probability of disease progression is 100% at 21 months in patients with high CIP2A protein. Interestingly, no correlation was observed between CIP2A expression and Sokal score, suggesting that the presence of high levels of this protein is an independent biomarker of blast crisis. In particular, of the six patients in this study who progressed to blast crisis only two had high Sokal scores (Table 7.1), while in the CCRe group, four of the fourteen patients had a high Sokal score but all achieved a CCRe.

CIP2A functions as a regulator of PP2A activity, and high expression of this protein is reported to suppress the phosphatase activity of PP2A.[271] Using specific siRNA and imatinib to reduce CIP2A levels in K562 cells resulted in a decrease in protein level of two important PP2A targets, c-Myc and PIM1. These proteins are important because PIM1 is a kinase that acts to phosphorylate c-Myc and stabilise its expression.[92,327] When PIM1 is dephosphorylated by PP2A it becomes degraded within the proteasome.[327] Similarly, c-Myc is also degraded in the proteasome once it is dephosphorylated by PP2A.[271] The reductions in PIM1 and c-Myc (and concomitant reduction of pS^{62}c-Myc) expression therefore suggest that PP2A activity increases. This notion is supported by the present observation that pY^{307}-PP2A levels decreased without affecting PP2A protein levels in the treated cells. Phosphorylation of PP2A on Y^{307} deactivates this phosphatase, and removal of this phosphate by PP2A-mediated auto-dephosphorylation results in its reactivation.[254] Taken together, the results of these experiments therefore suggest a mechanistic connection between high CIP2A expression, PP2A phosphorylation and suppression of activity (illustrated by high levels of pY^{307}-PP2A) and high levels of pS^{62}c-Myc and of c-Myc and PIM1 protein.

In the clinical samples analysed high CIP2A protein levels corresponded with high levels of Y^{307} and S^{62} phosphorylation of PP2A and c-Myc respectively. Moreover, the patient samples containing high levels of CIP2A also had high levels of c-Myc and PIM1. Taken together with the cell line findings, these data connect CIP2A to c-Myc and PIM1 in primary CML cells, and suggest that CIP2A, c-Myc and PIM1 could be prognostic biomarkers.

High levels of pS^{62}c-Myc and c-Myc protein may have important consequences. c-Myc has a critical role in cell proliferation, and increased levels of this protein within cells promote

entry into cell cycle. Thus, diagnostic chronic phase cells with high c-Myc are likely to have a greater proliferative potential. This notion is supported by studies of clinical gastric cancer biopsies in which aggressive (proliferative) disease is associated with high expression of CIP2A and c-Myc.[265] Deregulated cell division is known to result in increased DNA mismatch repair errors and in genomic instability.[96] This is also observed in CML, since c-Myc may contribute to disease progression by promoting aneuploidy.[96,97,320,321]

Whether CIP2A-mediated inhibition of PP2A acts directly to block dephosphorylation of c-Myc, or acts at a stage upstream of c-Myc remains to be determined. Junttila *et al* provide evidence suggesting that CIP2A binds directly to c-Myc and is recruited as a result of S^{62} phosphorylation.[271] However, this is complex since the reduction of CIP2A resulted in a decrease in BCR-ABL1 activity; this would then result in a decrease in the signals that are necessary to induce stable expression of c-Myc.

In the present study, patients from the CCRe group had high levels of SET protein in samples taken at diagnosis. At 12 month sampling, SET protein levels in the MNC from these patients were restored almost to normal levels. This is consistent with the *in vitro* findings of Neviani *et al,*[264] which demonstrated that PP2A is inhibited by SET, and that PP2A function can be restored by a reduction in SET levels caused by imatinib-induced inhibition of BCR-ABL1 and JAK2. Broadly similar data were seen in the No-CCRe group, who also did not undergo disease progression. However, patients in the BC group had lower SET levels than did patients who do not progress. This suggests that PP2A activity may be regulated differently in cells of CCRe and BC patients; in the former group of patients PP2A activity is regulated predominantly by SET, while in the latter group PP2A activity is regulated also by CIP2A.

Figure 8.10 summarises the present findings and postulates possible mechanisms of how PP2A is inhibited in CML. In patients who respond to imatinib therapy PP2A activity in the malignant cells is suppressed by SET, and SET expression is regulated by BCR-ABL1 via JAK2.[264] When imatinib is commenced, BCR-ABL1 activity decreases and this leads to removal of PP2A inhibition through a decrease in SET protein levels. However, in patients who progress to blast crisis CIP2A is present at a high level and contributes to (and may dominate) PP2A suppression. Imatinib therapy does not affect CIP2A levels in the malignant cells of these patients and PP2A remains suppressed. c-Myc expression therefore remains at a higher level, increasing the probability of subsequent genetic damage through increased cell proliferation and thus disease progression to blast crisis.

In summary, the present data demonstrate that CIP2A is an important determinant of future disease progression, and its use as a biomarker of blast crisis needs to be tested both in animal models and prospectively in clinical samples. Therapeutic targeting of CIP2A (which is a tumour associated protein) would be a more acceptable approach than targeting either PP2A or c-Myc which also have important functions in normal non-malignant cells.

CHAPTER NINE - General discussion

The aim of this thesis was to investigate whether patients destined to respond poorly to imatinib can be prospectively identified. Imatinib has undoubtedly revolutionised treatment for CML patients. However, at the time of starting this thesis the only data available on the efficacy of imatinib for newly diagnosed CML patients was from a single trial, IRIS, which was carried out under the supervision of the imatinib manufacturer Novartis. It was therefore important to establish if the IRIS trial outcome data could be reproduced in a more general, unselected CML population. The data presented in chapter three indicate that within our geographical area, following 24 months of imatinib treatment, the CCRe rate is 51%, meaning that nearly half of all patients will fail treatment by two years. This differs from the response rates seen within the IRIS study, which demonstrated that after five years of imatinib therapy the CCR rate was 87%.[18] The discrepancy between the present population study and the IRIS trial results may be explained in numerous ways. Firstly, there is a bias for low Sokal score patients within the IRIS trial. Secondly, there was an upper age limit and exclusion criteria for various concurrent medical conditions in IRIS, while in my population based study all adult patients were included, thus reflecting the typical range of patients seen in CML clinics on a regular basis. Thirdly, the IRIS trial censored patients at the point of imatinib discontinuation, thus with increasing follow-up the IRIS dataset becomes progressively selected for patients who have done well. The findings of this population study were later substantiated later by two independent studies.[287,328] In summary, half of all patients will fail imatinib treatment by two years, thus identification of prognostic markers predictive of treatment response may be useful in order to avoid delay in offering alternative treatment such as a second generation TKI or SCT.

The first biomarker investigated was the pCrkL/CrkL ratio as a surrogate marker for BCR-ABL1 tyrosine kinase activity (chapter four). The rationale behind this work was that imatinib is a TKI and thus an assay which measures the degree of BCR-ABL1 tyrosine kinase activity and how imatinib modifies this either *in vivo* or *in vitro* may offer additional prognostic information.

The assay demonstrated that FACS assessment of the pCrkL/CrkL ratio at diagnosis was predictive of achievement of a CCRe following 12 months of imatinib treatment. 100% of patients with an MFI of less than 25 achieved a CCRe, and importantly no patient lost their CCRe at any point, with a minimum follow-up of four years. No patient with an MFI above 25 achieved a CCRe following imatinib treatment; all subsequently required alterations in therapy which were either dose escalation or switching to a second generation TKI. Conversely all patients treated with nilotinib at initial diagnosis achieved a CCRe irrespective of their diagnostic pCrkL/CrkL ratio. No correlation was observed between the pCrkL/CrkL ratio and presenting WBC, Sokal score or hOCT1 mRNA expression levels, suggesting that the pCrkL/CrkL ratio is an independent biomarker of clinical outcome. These data suggest that the response to imatinib therapy is related to BCR-ABL1 tyrosine kinase activity present at diagnosis. A given dose of imatinib may therefore suppress the lower kinase activity to a greater proportional extent than the higher kinase levels in patients with an MFI above 25.[303]

However, there are some limitations with this assay. Firstly, a considerable amount of time was spent optimising the assay. One of the critical factors identified in the optimisation process was that the assay must be performed using fresh peripheral blood at diagnosis prior to any treatment. The logistics of getting a fresh blood sample to the laboratory for

assessment may pose some difficulties, thus it may only be possible to perform this assay at larger hospitals with a dedicated CML team. Furthermore, given that this study was performed on a small cohort of patients the findings need to be confirmed prospectively possibly as part of a large clinical trial. Nevertheless, in our laboratory it can be used to predict those patients who will achieve a CCRe and the technique is a valuable research tool.

Previous reports prior to the introduction of imatinib have in general, failed to identify any effect of *BCR-ABL1* transcript type on clinical outcome.[54-60] Chapter five investigated the role of *BCR-ABL1* transcript type on clinical outcome in the imatinib era. The major conclusions from the study were that patients with the e14a2 transcript may respond better to imatinib (400mg daily) than e13a2 patients, with the e14a2 patients exhibiting a superior time to achieve CCRe rate. Using the pCrkL/CrkL ratio at diagnosis as a surrogate for BCR-ABL1 tyrosine kinase activity,[197,303] it was determined that patients with the e13a2 transcript type had a higher BCR-ABL1 tyrosine kinase activity compared to e14a2 patients. It is therefore plausible that a standard dose of imatinib is only able to suppress the lower kinase activity i.e. that observed in the e14a2 patients and is insufficient at suppressing the higher kinase activity in e13a2 patients, thus e14a2 patients have a superior CCRe rate. The findings of this *BCR-ABL1* transcript type study were later substantiated by two independent studies conducted in Germany and Italy.[316,317]

There are many advantages of measuring *BCR-ABL1* transcript type compared to the pCrkL/CrkL ratio at diagnosis. Determination of *BCR-ABL1* transcript type is generally performed routinely at diagnosis in molecular diagnostic laboratories, thus the data are readily available and do not require a lengthy optimisation process or a special sample being

taken. However, the predictive power of the *BCR-ABL1* transcript type is not perfect. At 12 months 25% of patients with the e13a2 transcript and 53.8% of patients with the e14a2 transcript achieved a CCRe. These data suggest that patients with the e13a2 transcript type should be monitored carefully due to a high risk of imatinib failure. Knowledge of patient transcript type may therefore yield clinically useful data.[302]

The next potential biomarker to be investigated was *ALOX5*. The data provided by Chen *et al*[253] in a mouse model were interesting, and the fact that the data had not been substantiated in clinical samples made this an attractive area to pursue. As discussed in chapter six, the initial observations in primary CML samples were contradictory to the finding of Chen *et al.*[253] In the CML samples investigated *ALOX5* mRNA expression was down-regulated and not up-regulated as expected. Furthermore investigation of the *ALOX5* pathway, using LTB4 as a marker of *ALOX5* function (as performed by Chen *et* al[253]) revealed that there was an accumulation of LTB4 in the CML patients. LTB4 positively regulates *ALOX5,* therefore the results observed were puzzling. To investigate this further, the levels of the LTB4 receptor BLT1 were determined, since LTB4 can only positively regulate *ALOX5* by binding to its receptor BLT1. BLT1 protein in newly diagnosed patients was significantly lower than the level observed in patients responding to imatinib treatment and those patients who achieved a CCRe. The lack of BLT1 in CML patients allows the intermediates of the pathway, namely 5-HEPTE and LTA4, to suppress *ALOX5* gene expression and thus LTB4 accumulates as it has no receptor to bind to. The key finding here is that CML patients lack the LTB4 receptor BLT1, which is present in mice. The lack of BLT1 in CML patients means that the ALOX5 pathway studied in the CML mouse model does not function in the same way in human CML, which accounts for the difference observed between the clinical samples studied herein

and the Chen *et al*[253] study. At the start of this project, one of the potential long term aims was to use the *ALOX5* inhibitor zileuton in combination with imatinib to treat patients responding poorly to imatinib alone. Given that *ALOX5* is down-regulated in CML patients further inhibition would not be beneficial. These data suggest that we should be careful when extrapolating CML mouse model data into human CML.

The biomarker data presented in chapters four and five utilising the pCrkL/CrkL ratio or BCR-ABL1 transcript type can only predict patients likely to achieve a CCRe and cannot predict patients destined to subsequently progress into blast crisis.[302,303] Chapters seven and eight investigated the role of PP2A and its inhibitors SET, SETBP1 and CIP2A in CML. The critical findings of this work were that CIP2A protein is an important determinant of future disease progression in CML. High CIP2A protein levels were present in diagnostic MNC and CD34+ cells of patients destined to subsequently progress into blast crisis, and following imatinib treatment CIP2A levels increased further. The probability of disease progression was found to be 100% at 21 months in patients with high CIP2A protein. No correlation was observed between CIP2A expression and Sokal score, suggesting that the presence of high levels of this protein is an independent biomarker of blast crisis.

CIP2A functions as a regulator of PP2A activity, and high expression of this protein is reported to suppress the phosphatase activity of PP2A.[271] Using specific siRNA and imatinib to reduce CIP2A levels in K562 cells resulted in a decrease in BCR-ABL1 tyrosine kinase activity and restoration of PP2A activity and a decrease in the expression of two important PP2A targets, c-Myc and PIM1.

In the clinical samples analysed high CIP2A protein levels corresponded with high levels of Y^{307} and S^{62} phosphorylation of PP2A and c-Myc respectively. Moreover, the patient samples containing high levels of CIP2A also had high levels of c-Myc and PIM1. Taken together with the cell line findings, these data connect CIP2A to c-Myc and PIM1 in primary CML cells, and suggest that in addition to CIP2A, c-Myc and PIM1 could be prognostic biomarkers.

High levels of pS^{62}c-Myc and c-Myc protein may have important consequences. c-Myc has a critical role in cell proliferation, and increased levels of this protein within cells promote entry into cell cycle. Thus, diagnostic chronic phase cells with high c-Myc are likely to have a greater proliferative potential. Deregulated cell division is known to result in increased DNA mismatch repair errors and in genomic instability.[96] This is also observed in CML, since c-Myc may contribute to disease progression by promoting aneuploidy.[96,97,320,321]

At the time of conducting the CIP2A study, CIP2A had only been reported as a poor prognostic indicator in breast[93] and gastric[272] cancers. It has since been reported to be a poor prognostic indicator in tongue cancer,[329] NSCLC,[330] other lung cancer,[331] oral carcinoma and dysplasia,[332] oesophageal squamous cell carcinoma,[333] prostate cancer,[277] cervical cancer[334] and AML.[335] However, the mechanism by which CIP2A is up-regulated in cancer cells remains unknown.

Therapeutically targeting CIP2A, PP2A or c-Myc, in addition to BCR-ABL1 are all potential options to prevent disease progression. CIP2A, a tumour associated protein (arguably a

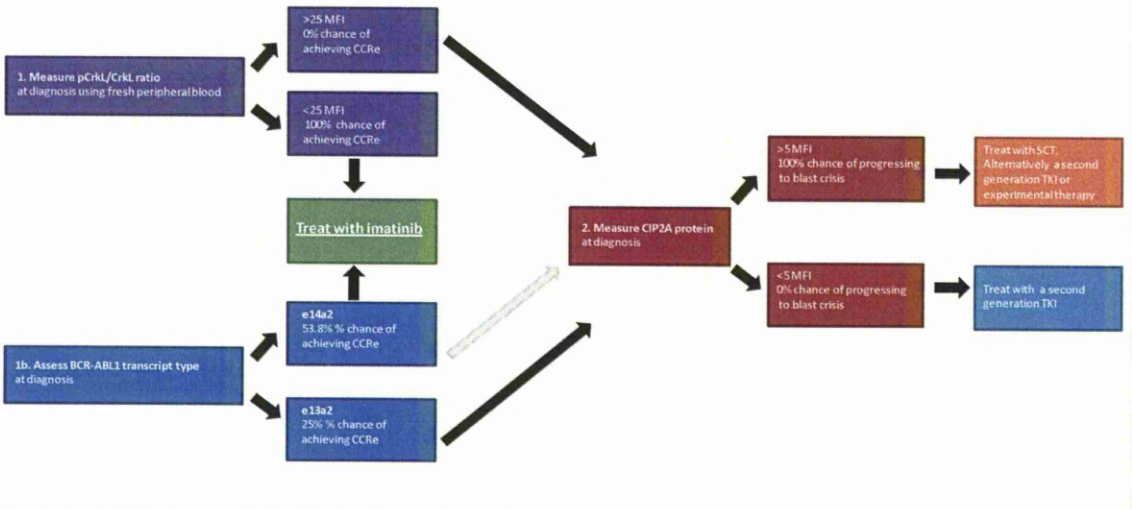
tumour specific protein), is probably the most attractive target, especially since it has been shown to play a role in other malignancies. There are many questions which remain unanswered with regard to CIP2A. Examples include: what causes it to be up-regulated in malignant cells? What is its normal function? What other proteins does it interact with? These questions need addressing before attempting to inhibit CIP2A function. An alternative approach would be to target either PP2A or c-Myc but this would need to be done cautiously as both are known to have normal functions in non-malignant cells.

PP2A is an important phosphatase in normal as well as malignant cells, with roles in cell growth and proliferation, DNA replication and repair, metabolism, neural signalling and apoptosis. Furthermore, PP2A protein exists as a trimeric complex consisting of a scaffolding subunit A, catalytic subunit C and a B subunit. Due to various isoforms of each subunit a variety of combinations are feasible, thus specifically targeting PP2A would be difficult. The PP2A inhibitor fostriecin has demonstrated cytotoxic effects in L1210 and P388 mouse lymphocytic leukaemia cell lines. Fostriecin was tested in a clinical trial but was found to be quite toxic and no clinical benefits were observed.[336] FTY720 is an immunomodulating drug approved for treating multiple sclerosis and has been reported to reactivate PP2A in CML cell lines.[337] However, FTY720 is inactivated when phosphorylated, and unfortunately *in vitro* data suggest that PP2A inhibition in CML patients would require a dose far greater than that which can practically be achieved. Therefore, targeting PP2A directly may be extremely difficult and thus targeting its inhibitory proteins may be more feasible option.

c-Myc expression is essential for all proliferating cells. The majority of malignant cells are c-Myc dependent and require continuous c-Myc expression to sustain tumour proliferation and

viability.[338] In 20 human cancer cells lines, shRNA-mediated depletion of c-Myc resulted in permanent proliferative arrest, with cells accumulating in S or G2/M phases. Inhibition of c-Myc in normal cells resulted in G_0/G_1 arrest.[339] c-Myc knock-out mice die *in utero* at E9.5 and 10.5, exhibiting pleiotropic phenotypes including small size, developmental delay, enlarged heart and pericardium, decreased yolk sac circulation, and aberrant neural tube closure.[340] In 2002 the embryonic lethality of c-Myc knock-out mice was attributed to defects in vasculogenesis, angiogenesis, and primitive erythropoiesis in the yolk sac and in the embryo proper.[341] A major concern with an anti-cancer strategy involving c-Myc inhibition would be the toxic effects on normal cells. Nevertheless, most normal cells are quiescent and express very little c-Myc, so it is plausible that a c-Myc inhibitor might have little effect on normal cells.

Figure 9.1 Biomarker flow chart



In this thesis numerous potential biomarkers have been identified. Some biomarkers predict the achievement of a CCRe while others predict disease progression, however no single biomarker can predict cytogenetic response and disease progression. Figure 9.1 shows a biomarker flow chart and suggests when each test should be performed. At diagnosis the pCrkL/CrkL ratio can predict achievement of a CCRe. If a patient has a pCrkL/CrkL ratio MFI less than 25 then that patient is likely to achieve a CCRe while on imatinib treatment and no further tests are required. If however, a patient has a pCrkL/CrkL ratio MFI greater than 25 then this patient will not achieve a CCRe but whether or not the patients will progress into blast crisis cannot be determined using the pCrkL/CrkL ratio. To determine if a patient is at a high risk of disease progression assessment of CIP2A protein level at diagnosis will identify those patients who will subsequently progress into blast crisis, for whom a more aggressive treatment strategy may be appropriate. If however, assessment of the pCrkL/CrkL ratio is not possible then knowledge of the *BCR-ABL1* transcript type could provide additional information. The prognosis is poor for patients with the e13a2 transcript type, these patients have only a 25% chance of achieving a CCRe. Therefore, these patients should have their CIP2A status assessed at diagnosis in order to determine if they are at risk of future disease progression.

Numerous biomarkers have been identified in CML by other groups, as discussed throughout this thesis and are summarised in Table 9.1. Some proteins are elevated at disease progression but at diagnosis offer no prognostic value. The clinical value of a protein which is either up or down-regulated as a patient progresses is small, as this information could be provided by simple methods such as looking at the FBC or morphology, or by serial trends in BCR-ABL1 quantitation or marrow cytogenetics. Proteins or genes which are differentially expressed at diagnosis and have been associated with clinical outcome post imatinib

treatment make ideal biomarkers. Many of the biomarkers reported to predict a patient's clinical outcome have been tested on a limited cohort of patients. Nevertheless, these biomarkers have shown significant promise and warrant further investigation as part of a large clinical trial. Table 9.1 lists the current biomarkers and whether or not, in my opinion, further investigation of them would be worthy.

Table 9.1 Summary of identified biomarkers in CML

Biomarker	Discussed in section	Candidate for further investigation	Reasoning
CIP2A [94]	1.8.6 and 8.2.0	Yes	This novel biomarker of blast crisis needs to be confirmed on a larger cohort.
c-Myc[94]	1.4.3 and 7.3.8	Yes	This biomarker, with its serine 62 phosphorylation status needs to be confirmed on a larger cohort.
PIM1[92,94,274]	1.8.7 and 8.2.6	Yes	This biomarker needs to be confirmed on a larger cohort using CD34+ cells.
PP2A[94]	1.8.3 and 7.3.0	Yes	This biomarker needs to be confirmed on a larger cohort using both MNC and CD34+ cells.[94]
pCrkL/CrkL ratio[197,303]	1.6.8 and 4.3.0	Yes	This needs to be tested prospectively using fresh MNC.
Additional chromosome abnormalities[82]	1.4.3	Yes	Considered markers of disease progression and an indication of genomic instability.
***BCR-ABL1* transcript type**[302,317316]	1.3.4 and 5.3.0	Yes	This needs to be tested prospectively.
Eutos score[160]	1.6.2.1	Yes	This novel score needs to be tested prospectively as part of a clinical trial.
Radich gene array signature[228]	1.6.9.1	Yes	This could be assessed as part of a clinical trial.
TWIST1[223]	1.6.9.2	Yes	Potentially novel biomarker of imatinib resistance.[223]

Yong gene array signature[206]	1.6.9.1	Yes	This needs to be tested prospectively using MNC and CD34+ cells..
ALOX5	1.7.1 and 6.3.0	No	Offers no prognostic value in CML.
BCR-ABL1 KD mutations	1.6.7	No	Mutations are rarely detected at diagnosis and not all mutations confer drug resistance.
CEBPα and hnRNPE2	1.4.5	No	Offers no prognostic value, increases during blast crisis, but has not been tested prospectively. [108,218]
EVI-1[212]	1.6.9.1	No	EV1-1 expression predicts clinical outcome in imatinib resistant patients treated with a second generation TKI.[212]
hOCT1[147,166,342,343]	1.6.5	No	Multiple studies have confirmed the prognostic value of hOCT1[147,166,342,343]
JAK2[216]	1.4.1	No	Influenced by other proteins in addition to BCR-ABL1.[216,344-346]
Plasma monitoring[173-176]	1.6.6	No	Data suggest that a number of variables can influence the plasma level of imatinib.[173-176]
SET[94,264]	1.8.4 and 7.3.7	No	Offers no prognostic value, increases during blast crisis.[94,264]
SETBP1[94,265,347]	1.8.5 and 7.3.7	No	Offers no prognostic value in CML.[94,347]
Sokal Score[155]	1.6.1.1 and 3.3.3	No	Data suggest that Sokal score is a poor prognostic indicator in imatinib treated patients[21,160]

STAT5[74,75]	1.4.1	No	Not immediately downstream of BCR-ABL1.[348,349]
WT1[213]	1.6.9	No	Offers no prognostic value, increases during blast crisis.[213]

The prime biomarker which warrants further investigation is CIP2A. CIP2A is the only biomarker reported at diagnosis to predict those patients who will subsequently progress into blast crisis.[94] However, the present data are confined to patients treated with imatinib from original diagnosis. It remains to be seen whether its predictive value holds for patients treated first-line by a more potent TKI such as nilotinib or dasatinib. c-Myc, PIM1, PP2A and the pCrkL/CrkL ratio have all been shown to indicate patients destined to fair poorly on imatinib treatment.[94,303] It is possible however that changes observed in c-Myc, PIM1, PP2A and pCrkL/CrkL ratio are simply a reflection of CIP2A activity and are therefore not independent biomarkers. Potentially assessing CIP2A at diagnosis may be the only parameter required to determine if a patients is at risk of disease progression.

The original aims of this thesis have been addressed as follows:

1. **To establish if IRIS and other clinical trial data can be extrapolated to an unselected CML population. This will be done by determining 'real' rates of CCR and MMR, and thus the rate of imatinib failure, for patients treated within our region.**

   This has been addressed in chapter three. In an unselected CML population, the dramatic results of the IRIS clinical trial are still seen but the 'real' rates of CCR and MMR are much lower..

2. **To evaluate recently reported biomarkers for CML outcome for their significance in determining outcome of modern CML therapy:**

   a. **Whether BCR-ABL1 tyrosine kinase activity can predict outcome.**

   Assessing the BCR-ABL1 tyrosine kinase activity at diagnosis as described in chapter four can identify those patients who will achieve a CCRe following 12 months of imatinib treatment.

   b. **Whether the BCR-ABL1 transcript type affects outcome.**

   The *BCR-ABL1* transcript type provides additional clinical information. Patients with the e13a2 transcript type have an inferior response to imatinib compared to e14a2 patients. This is due to e13a2 patients having much higher BCR-ABL1 tyrosine kinase activity, as shown in chapter five.

3. **To investigate new biomarkers for their predictive value on outcome.**

   a. **Investigate the role of *ALOX5* in human CML for the first time.**

      ALOX5 expression or function provides no prognostic clinical information. This is attributed to differences in the *ALOX5* pathway in humans and CML mouse models, as discussed in chapter six.

   b. **Investigate the role of PP2A and its inhibitory proteins (SET, SETBP1 and CIP2A) in CML cells and ascertain whether any /all are predictive of clinical outcome.**

      CIP2A is a novel biomarker of blast crisis in CML as described in chapter eight. Assessment of SET or *SETBP1* at diagnosis was not predictive of clinical outcome in the CML patients investigated. PP2A, c-Myc and PIM1 protein expression were also indicative of a patient's eventual clinical outcome.

In conclusion, in identifying patients likely to achieve a CCRe on imatinib treatment the most promising biomarker investigated was the pCrkL/CrkL ratio. 100% of patients with an MFI less than 25 achieved a CCRe. If assessment of the pCrkL/CrkL ratio was not possible then alternatively a patient's *BCR-ABL1* transcript type can provide additional information. Identification of patients who will subsequently progress into blast crisis on imatinib treatment is now possible, by assessment of the CIP2A protein at diagnosis. Collectively, the biomarkers studied in this thesis can potentially predict the outcome of CML patients treated with imatinib first-line.

APPENDIX - Publications arising from this thesis

Cancerous inhibitor of PP2A (CIP2A) at diagnosis of chronic myeloid leukemia is a critical determinant of disease progression

Claire M. Lucas,[1] Robert J. Harris,[1] Athina Giannoudis,[1] Mhairi Copland,[2] Joseph R. Slupsky,[1] and Richard E. Clark[1]

[1]Department of Haematology, University of Liverpool, Liverpool, United Kingdom; and [2]Paul O'Gorman Leukaemia Research Centre, College of Medical, Veterinary and Life Sciences, University of Glasgow, Gartnavel General Hospital, Glasgow, United Kingdom

Prospective identification of patients whose chronic myeloid leukemia (CML) will progress to blast crisis is currently not possible. PP2A is a phosphatase and tumor suppressor that regulates cell proliferation, differentiation, and survival. Cancerous inhibitor of PP2A (CIP2A) is a recently described inhibitor of PP2A in breast and gastric cancer. The aim of this study was to investigate whether CIP2A played a role in CML and whether PP2A or its inhibitor proteins CIP2A or SET could predict clinical outcome. At the time of diagnosis of CML, patients who will later progress to blast crisis have significantly higher levels of CIP2A protein ($P < .0001$) than patients who do not progress, suggesting that PP2A is functionally inactive. We show that the potential mechanism for disease progression is via altered phosphorylation of the oncogene c-Myc. Knockdown of CIP2A results in increased PP2A activity, decreased c-Myc levels, and a decrease in BCR-ABL1 tyrosine kinase activity. We demonstrate that CIP2A levels at diagnosis can consistently predict patients who will progress to blast crisis. The data show that CIP2A is biologically and clinically important in CML and may be a novel therapeutic target. (*Blood*. 2011;117(24):6660-6668)

Introduction

Chronic myeloid leukemia (CML) is a malignant disease of the primitive hematologic cell, characterized by inappropriate expansion of myeloid cells. Although this disease is readily controlled by imatinib, approximately one-third of patients will eventually fail treatment[1,2]; and a significant proportion of these will progress toward blast crisis (BC), which is usually rapidly fatal. Poor response to imatinib and progression to BC have been linked to high BCR-ABL1 tyrosine kinase activity,[3] but why one patient can remain in well-controlled chronic phase for decades whereas another may rapidly progress to BC is poorly understood.

The BCR-ABL1 tyrosine kinase in CML is responsible for growth and survival of the malignant cells through activation of signaling pathways, such as the mitogen-activated protein kinase cascade and the PI3K pathway.[4,5] A major cellular serine/threonine phosphatase working to down-regulate activation of these pathways is the tumor suppressor protein phosphatase 2A (PP2A).[6] In CML cells, PP2A is a key target of BCR-ABL1 signaling; this protein becomes inactivated in these cells because BCR-ABL1 stimulates prevention of its auto-dephosphorylation at tyrosine^{307}.[7,8] Maintenance of pY^{307}-PP2A levels in CML cells feeds back to BCR-ABL1 and facilitates increased and sustained kinase activity. Inhibition of BCR-ABL1 by imatinib results in reactivation of PP2A, inducing both suppression of growth and enhanced apoptosis of the leukemic cells.[8] However, it is unknown whether Y^{307} in PP2A is a direct substrate of BCR-ABL1, and the mechanism regulating phosphorylation of PP2A at this site is not clearly defined.

One proposal for the mechanism through which BCR-ABL1 regulates PP2A activity in CML cells involves expression of the PP2A inhibitor protein SET. In CML cell lines, increased expression of BCR-ABL1 increases expression of SET through a process that is mediated by JAK2.[9] Furthermore, Neviani et al[8] also observed that SET levels rose at evolution of BC in chronic phase and BC primary CML samples, and that incubation with imatinib decreased SET levels in primary CML cells from a single patient undergoing BC.

Two additional proteins have been shown to inhibit PP2A activity. Overexpression of SET binding protein 1 (SETBP1) protects SET from protease cleavage and permits formation of a SETBP1-SET-PP2A complex, which inhibits PP2A phosphatase activity. However, although one report has indicated that high levels of *SETBP1* expression predict adverse outcome in elderly patients with acute myeloid leukemia (AML),[10] we have recently demonstrated that *SETBP1* expression does not correlate with clinical outcome in CML.[11] Thus, *SETBP1* expression is probably not the inhibitory block on PP2A function in CML. The second protein of interest, called cancerous inhibitor of PP2A (CIP2A), functions in a cancer setting to prevent PP2A-mediated dephosphorylation of c-Myc at serine 62. pS^{62}-Myc is stabilized against degradation, and CIP2A therefore promotes deregulated cell growth.[12] High expression levels of CIP2A correlate with aggressiveness of breast and gastric cancers,[13-15] and CIP2A depletion decreases growth of cells from these cancers.[13,15] In mouse neural progenitor cells, overexpression of CIP2A increases progenitor cell self-renewal and proliferation.[16] CIP2A has recently been shown to be overexpressed in AML patients and correlated with relapse after treatment.[17] A single case study has reported a CIP2A-MLL translocation in infant AML.[18] Nothing is known about CIP2A expression and function in CML.

Submitted August 28, 2010; accepted March 31, 2011. Prepublished online as *Blood* First Edition paper, April 13, 2011; DOI 10.1182/blood-2010-08-304477.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

Table 1. Patient characteristics

	No. of patients	Male/female	Average age, y	Sokal score			
				Low	Intermediate	High	Unknown
CCR	14	7/7	45	6	1	4	3
No-CCR	11	7/4	48	2	2	5	2
BC	6	5/1	37	1	2	2	1
Total	31	31	44	9	5	11	6

CCR indicates complete cytogenetic response; No-CCR, patients who had achieved a complete hematologic response but not a CCR by 12 months and who had not progressed; and BC, patients presented in CP but subsequently progressed to BC.

Here we have investigated the expression of PP2A and its inhibitory proteins SET and CIP2A in CML patients at diagnosis and at 12 months after diagnosis or at disease progression. We show that the levels of these proteins are distinctly different according to the later outcome of the patient. In particular, we identify CIP2A as a potential predictive biomarker of disease progression because CIP2A protein levels are significantly higher in CML patients who later progress to BC than in patients who do not. Furthermore, we also show that high CIP2A levels in primary CML cells correlate with high levels of pS^{62}-Myc, suggesting a link between high CIP2A expression, increased growth potential, and genetic instability. Finally, we support our clinical observations with mechanistic data; we show that small interfering RNA (siRNA)–mediated knockdown of CIP2A expression in K562 cells results in increased PP2A activity, decreased c-Myc levels, and a decrease in BCR-ABL1 tyrosine kinase activity. To our knowledge, this is the first report of CIP2A dictating outcome in any hematologic malignancy.

Methods

Patient cohort

All 31 CML patients included in this study were diagnosed in chronic phase at our center. The study was approved by the Liverpool Local Research Ethics Committee, and informed consent in accordance with the declaration of Helsinki was obtained from each patient. All were 18 or more years of age at diagnosis and were positive by metaphase cytogenetic analysis for the t(9;22) (Philadelphia) translocation. Quantitative RT-PCR was used to quantify *BCR-ABL1* gene fusion transcripts as previously described.[19] Patient characteristics are described in Table 1. All cases started imatinib treatment within 4 weeks of first presentation.

Clinical response

Patients were stratified into 3 clinical outcomes:

CCR. Complete cytogenetic response (CCR) = no Philadelphia-positive metaphases among at least 20 marrow metaphases after 12 months of imatinib treatment. In some cases, serial cytogenetic data were not available, and achievement of CCR is based on a BCR-ABL1/ABL1 transcript ratio of $< 1\%$, which we have previously shown[20] to be tightly correlated with cytogenetically defined CCR (n = 14).

No-CCR. Patients who had achieved a complete hematologic response but not a CCR by 12 months and who had not progressed (n = 11).

BC. Patients presented in chronic phase but who subsequently progressed into BC (n = 6).

Sample collection and preparation

Mononuclear cells (MNCs) were separated from peripheral blood at diagnosis and after 12 months of imatinib treatment or at disease progression, by density-dependent centrifugation (Lymphoprep Axis-Shield), washed in RPMI 1640 (BioSera), and resuspended in 10% DMSO/10% FCS serum (BioSera)/RPMI at 4°C and cryopreserved in liquid nitrogen. When required, the cells were thawed and resuspended in culture media as previously described.[19] K562, LAMA84, KCL22, and KY01 cell lines were used as BCR-ABL1-positive cell lines. The gastric cancer cell line AGS was used as a CIP2A-positive BCR-ABL1-negative control.

Samples were enriched for $CD34^+$ cells using CliniMACS (Miltenyi Biotec) according to the manufacturer's instructions. All $CD34^+$ cells were collected at diagnosis and then grouped according to the patients' eventual clinical outcome: CCR (n = 3), No-CCR (n = 4), or BC (n = 3).

Measurement of PP2A, phosphorylated PP2A, SET, CIP2A, and PIM1

Flow cytometric assays were used for the detection of PP2A, phosphorylated PP2A, SET, CIP2A, and PIM1 as previously described,[3] using anti-PP2A antibody (Millipore), PP2A Y307 (Epitomics), SET (Santa Cruz Biotechnology), CIP2A (Santa Cruz Biotechnology), PIM1 (Abcam), and anti–mouse and anti–rabbit AlexFluor-488 (Invitrogen). Supplemental Figure 1 (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article) shows the optimization of the flow cytometry assay. Western blotting was used to confirm all findings in both K562 and LAMA84 CML cell lines.

Measurement of CrKL phosphorylation

Levels of pCrKL and CrKL (CT10 regulator of kinase-like) were measured by FACS as previously described.[3]

TaqMan gene expression assay of PP2A (catalytic subunit), SET, and SETBP1

Predesigned TaqMan quantitative RT-PCR assays were used in a 384-well assay plate. Each assay consists of a forward and reverse primer at 900nM each and a 6-FAM dye-labeled TaqMan MGB probe at 250nM. The assay's ID numbers are: *PP2A* (catalytic subunit), Hs00427259m1, *SET* Hs00853870g1, *SETBP1* Hs00210209m1, and *GAPDH* Hs99999905m1. The relative expression level was calculated by the comparative C_t method that uses the $2^{-\Delta\Delta Ct}$ formula to achieve results for relative quantification.[21]

CIP2A mRNA expression

The expression of *CIP2A* was measured using quantitative RT-PCR on a LightCycler 1.5 using LightCycler FastStart DNA MasterPlus SYBR Green I (Roche Diagnostics). CIP2A primers and PCR conditions were as previously described.[13]

c-Myc protein and phosphorylation status

Whole-cell lysates were prepared from MNCs, using NP40 lysis buffer. ELISA methodology was adapted from Hamilton et al[22] using anti-Myc ser62 antibody[23] (Abcam) and anti-Myc antibody (New England Biolabs).

CIP2A siRNA treatment

A total of 1×10^6 K562 cells were resuspended in 100-μL solution V Nucleofector Kit V (Amaxa Biosystems); 100nM siRNA was added to solution V (CIP2A siRNA and control siRNA; Santa Cruz Biotechnology). In each case, samples were nucleofected using the Amaxa instrument preset program T16. After nucleofection, the cells were cultured for 72 hours in a 24-well plate before analysis. In addition, these experiments were repeated

using a different CIP2A siRNA (Integrated DNA Technologies) and a different CML cell line LAMA84.

Statistical analysis

Statistical analysis and comparisons were performed by Mann-Whitney and Student *t* tests using SPSS Version 16.0 (SPSS).

Results

PP2A protein expression and phosphorylation are increased in MNCs and CD34+ cells from patients destined to progress to BC

In initial experiments, PP2A protein levels were compared between chronic phase and established BC. Significantly elevated levels were seen in BC cells (supplemental Figure 2).

To determine whether PP2A protein levels were indicative of disease progression, we analyzed PP2A protein in MNCs of 31 patients who were in chronic phase at diagnosis and again in the same patients after 12 months of treatment or at transformation. The clinical response of these patients was stratified into 3 groups depending on outcome: CCR, No-CCR, and BC. No BCR-ABL1 kinase domain mutations were detected in any patients. At diagnosis, the mean PP2A protein level in patients destined to achieve CCR was not significantly different from that in normal MNCs, whereas that in patients destined not to achieve CCR (No-CCR) was significantly lower than that in normal cells ($P = .02$; Figure 1A). In both groups of patients, PP2A levels increase after 12 months of treatment. In contrast to the CCR and No-CCR patients, PP2A levels in patients destined to progress into BC were much higher ($P < .001$ compared with the No-CCR group), and these levels did not change after treatment (Figure 1A). When the same experiment was performed on CD34+ cells from the same patients, similar results were obtained (Figure 1B); PP2A protein was elevated in the diagnostic CD34+ cells from patients who subsequently progress into BC compared with the CCR and No-CCR groups. These results suggest that increased PP2A protein expression at diagnosis indicates a high probability of disease progression.

We next investigated whether PP2A protein levels were a function of PP2A gene expression. Quantitative RT-PCR was used to measure expression of the gene coding for the PP2A catalytic subunit. The pattern of PP2A gene expression was similar to that of PP2A protein expression. Interestingly, PP2A gene expression in all 3 groups, regardless of whether the sample was taken at diagnosis or after treatment, was lower than that observed in normal cells. Because protein levels of PP2A in the MNCs from the BC group were higher than in normal MNCs, this suggests a role for posttranslational modification in the stabilization of PP2A protein expression in the BC group (supplemental Figure 3).

PP2A is inactive when phosphorylated at tyrosine 307, and this can be used to indicate its activity level.[8,10,24] Phosphorylated levels of PP2A were significantly greater at diagnosis in the CCR ($P = .01$), No-CCR ($P = .002$), and patients who progressed into BC ($P = .012$) and at BC ($P = .004$), compared with normal MNCs (Figure 1C). We next analyzed PP2A phosphorylation status in CD34+ cells from patients in these response groups and found elevated levels of pY^{307}-PP2A in cells from the CCR and No-CCR response groups but dramatically increased levels of this phosphoprotein in cells from patients destined to progress (Figure 1D). These data support the conclusions of a previous report indicating a role for PP2A inactivation in the pathology of CML.[8] Taken together, these data demonstrate that PP2A proteins and phosphorylation levels are increased in the MNCs and CD34+ cells of patients destined to progress to BC.

SET and SETBP1 expression is not indicative of clinical outcome

SET and SETBP1 have, respectively been implicated in regulating PP2A activity in the leukemic cells of CML and AML.[8,10] We therefore measured the expression of *SET* and *SETBP1* at diagnosis and again after 12 months of treatment or at transformation. In line with our previously reported data on *SETBP1* expression,[11] we found that expression of *SETBP1* did not differ between the CCR, No-CCR, and BC clinical groupings of CML (Figure 2A). Moreover, and unlike in the leukemic cells of AML,[10] *SETBP1* gene expression levels were lower in CML samples compared with those from normal subjects. Interestingly, SETBP1 expression increased after 12 months of treatment in the CCR and No-CCR groups.

SET protein levels (Figure 2B) were significantly higher at diagnosis in the CCR and No-CCR groups compared with normal ($P < .001$ and $P = .01$, respectively). In patients who subsequently progressed to BC, there was a trend for lower SET levels. This trend was also seen for *SET* mRNA levels (Figure 2C) because samples from patients who later progressed had lower levels of *SET* mRNA than did samples from either CCR or No-CCR patients. Interestingly, in BC samples at transformation, SET protein levels increased to become spread over a higher range than at diagnosis and may suggest that SET plays a role during disease progression. This observation contrasts with the fall in *SET* expression observed in samples from patients within the CCR and No-CCR groups after 12 months of treatment. When we examined SET protein expression in diagnostic CD34+ cells, there was no apparent difference between the 3 clinical groups, although all patients were receiving imatinib treatment at 12 months or at disease progression follow-up (Figure 2D). Taken together, these results are broadly in agreement with previously reported data[8] but also suggest that SET may not be the only factor inhibiting PP2A in the malignant cells from patients destined to progress to BC (compared with those from patients who do not). This implies that another mechanism may be inhibiting PP2A in this group of patients.

CIP2A protein level in MNCs and CD34+ at diagnosis is predictive of BC

An additional protein shown to regulate PP2A activity in some cancers that may have a role to play in CML is CIP2A.[12-14] Figure 3A shows that CIP2A gene expression is not significantly different in any of the 3 response groups from that in normal MNCs. No statistically significant difference was observed between any of the 3 response groups or at either time point, nor were any of these different from normal MNCs.

In sharp contrast, Figure 3B demonstrates that CIP2A protein levels in MNC taken at diagnosis were significantly higher in patients who later progressed to BC than in patients from the CCR and No-CCR groups ($P < .0001$ and $P = .01$, respectively). This observation was also confirmed in CD34+ cells; samples from patients destined to progress into BC had much higher levels of CIP2A than either CCR or No-CCR patients (Figure 3C). No correlation was seen between CIP2A expression and Sokal score (data not shown). These results suggest that CIP2A may be important in inhibiting PP2A function in CML patients who progress to BC. The importance of increased CIP2A protein

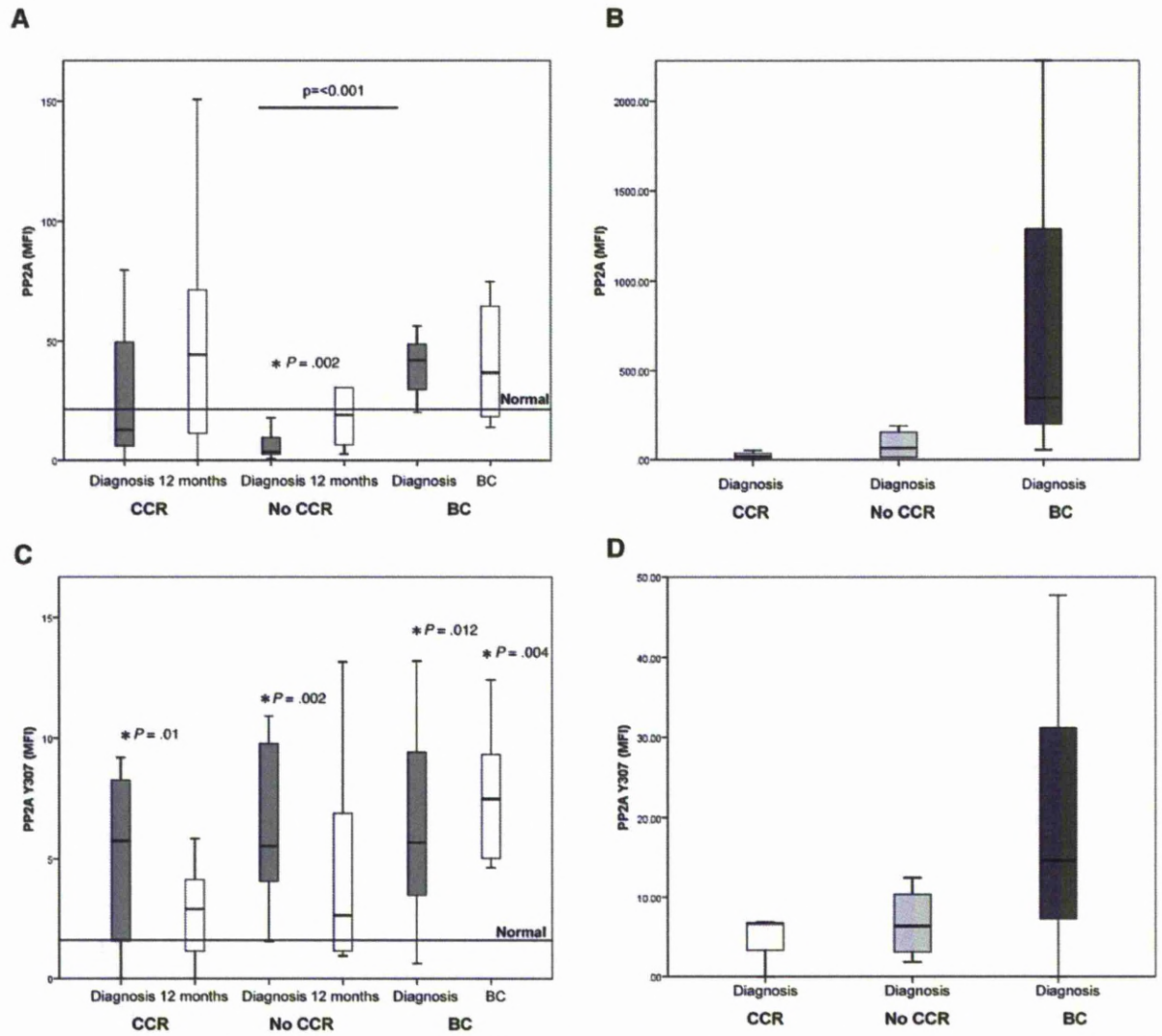


Figure 1. PP2A expression and phosphorylation are increased in MNC and CD34⁺ cells from patients destined to progress to BC. The horizontal line represents the mean normal level observed in 10 healthy volunteers. (A) Levels of PP2A protein as assessed by flow cytometry. Mean level in 10 healthy subjects = 21.4 (range, 17.1-25.7). The level of PP2A is high in patients destined to progress to BC, compared with nonresponding patients ($P = .001$). *Statistically significant difference between No-CCR and normal ($P = .002$). CCR, n = 14; No-CCR, n = 11; and BC, n = 6. (B) Level of PP2A protein in CD34⁺ cells. The level of PP2A is high in CD34⁺ cells in patients destined to progress to BC. CCR, n = 3; No-CCR, n = 4; and BC, n = 3. (C) Level of phosphorylated (inactive) PP2A protein, as assessed by flow cytometry (mean level in 10 healthy subjects = 1.6; range, 0.8-2.3). Phosphorylated levels of PP2A were greater at diagnosis in CCR ($P = .01$), No-CCR ($P = .002$) patients who progressed into BC ($P = .012$), and at BC ($P = .004$) compared with normal MNCs. CCR, n = 14; No-CCR, n = 11; and BC, n = 6. (D) Level of phosphorylated (inactive) PP2A protein in CD43⁺ cells. The degree of phosphorylation is greater in the CD34⁺ from patients destined to progress into BC. CCR, n = 3; No-CCR, n = 4; and BC, n = 3.

expression in disease progression is further exemplified after 12 months of treatment; whereas CIP2A protein levels remain essentially unchanged in samples from the CCR and No-CCR groups, the level increases further at disease progression ($P = .008$, Figure 3B).

To test whether high expression of CIP2A protein is predictive of disease progression, progression-free survival was compared between patients with high (defined as a mean fluorescence intensity > 5) and low diagnostic CIP2A protein levels. Figure 3D shows that the median time to disease progression in patients with high CIP2A protein levels is 13 months, and their probability of progression is 100% at 21 months. Five of these 6 patients have died of their progression. In contrast, during the same time period, none of the patients with low diagnostic CIP2A levels progressed, and all remain progression-free and alive at their latest follow-up (average follow-up, 47 months). These data indicate that CIP2A protein expression is a biomarker of disease progression in CML.

We have previously demonstrated that BCR-ABL1 activity levels are also predictive of treatment response in CML.[3] To see whether BCR-ABL1 was a factor controlling CIP2A protein levels, we treated the BCR-ABL1-positive cells lines K562, KCL22, and KY01 together with the BCR-ABL-negative gastric cancer cell line AGS (used as a CIP2A positive control)[14] with the tyrosine kinase inhibitor imatinib for 24 hours. We found that imatinib treatment of the BCR-ABL1-positive cell lines resulted in a significant down-regulation of CIP2A protein levels ($P = .002$ for K562, $P = .001$

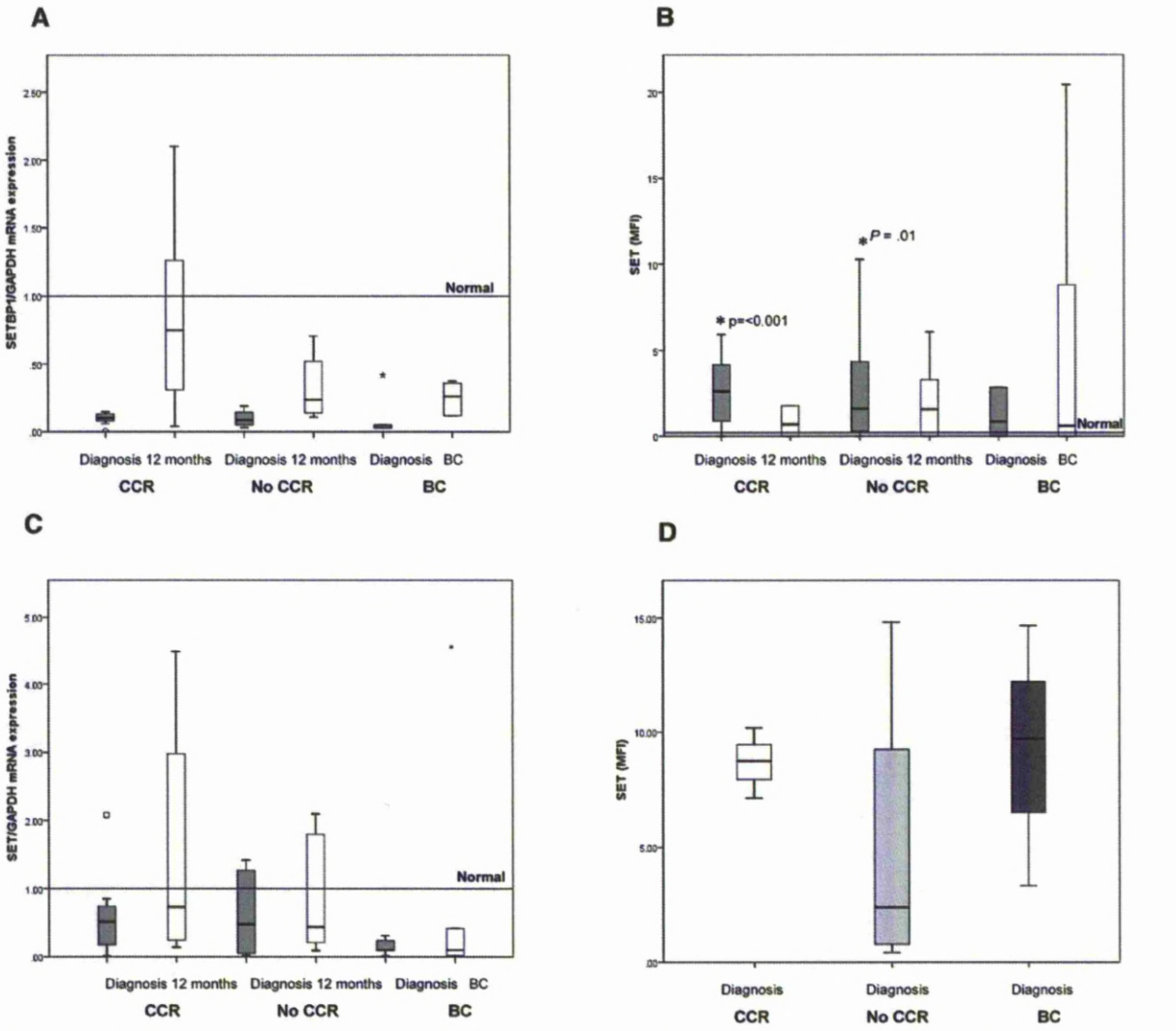


Figure 2. SET and SETBP1 at diagnosis and follow-up. SET protein is low at diagnosis in patients who subsequently progress to BC. (A) mRNA expression of SETBP1. No significant differences in *SETBP1* expression were observed between the 3 response groups. CCR, n = 14; No-CCR, n = 11; and BC, n = 6. (B) Levels of SET protein. SET protein levels are higher than in normal MNCs (mean level in 10 healthy subjects = 0.2; range, 0.1-0.3) in the CCR and No-CCR groups ($P < .001$ and $P = .01$, respectively). CCR, n = 14; No-CCR, n = 11; and BC, n = 6. (C) SET mRNA expression. CCR, n = 14; No-CCR, n = 11; and BC, n = 6. (D) Level of SET protein in $CD34^+$ cells. The level of SET protein assessed at diagnosis does not correlate with the patients' eventual clinical outcome, although this outcome is based on only 3 patients who later progressed while on imatinib treatment. CCR, n = 3; No-CCR, n = 4; and BC, n = 3.

for KCL22, and $P = .024$ for KY01) but had no effect on CIP2A expression in AGS cells. Thus, BCR-ABL1 activity may be an important factor in the regulation of CIP2A protein expression (Figure 3E).

c-Myc protein is elevated at diagnosis in patients destined to progress to BC

One of the prescribed functions of CIP2A in cancer cells is to prevent PP2A-mediated dephosphorylation of c-Myc.[12] To test whether c-Myc phosphorylation is a factor in CML pathophysiology, we used an ELISA to measure the levels of pS^{62}-Myc in diagnostic samples. MNCs from patients destined to progress to BC had significantly higher levels of pS^{62}-Myc than did those from patients within either the CCR or No-CCR groups (Figure 4A; $P = .04$). Because S^{62} phosphorylation of c-Myc stabilizes this protein against degradation,[12] we also measured levels of total c-Myc protein in the same sample. We observed similar results; c-Myc protein levels were significantly higher in the cells of patients who subsequently progress to BC (Figure 4B; $P = .002$). These data demonstrate that high levels of c-Myc and phosphorylation at serine 62 indicate a high risk of disease progression. Because PP2A is the major phosphatase that dephosphorylates c-Myc, these results further suggest that PP2A function may be inhibited, possibly by high expression of CIP2A.

PIM1 is a protein kinase that can phosphorylate and stabilize c-Myc.[23] PIM1 is itself phosphorylated, and in this state it is active and its expression is stable.[25] However, this protein is a target for PP2A; and once it is dephosphorylated by PP2A, it is rapidly degraded in the proteasome.[26] To further confirm that PP2A is functionally inactive in CML, we investigated PIM1 as another

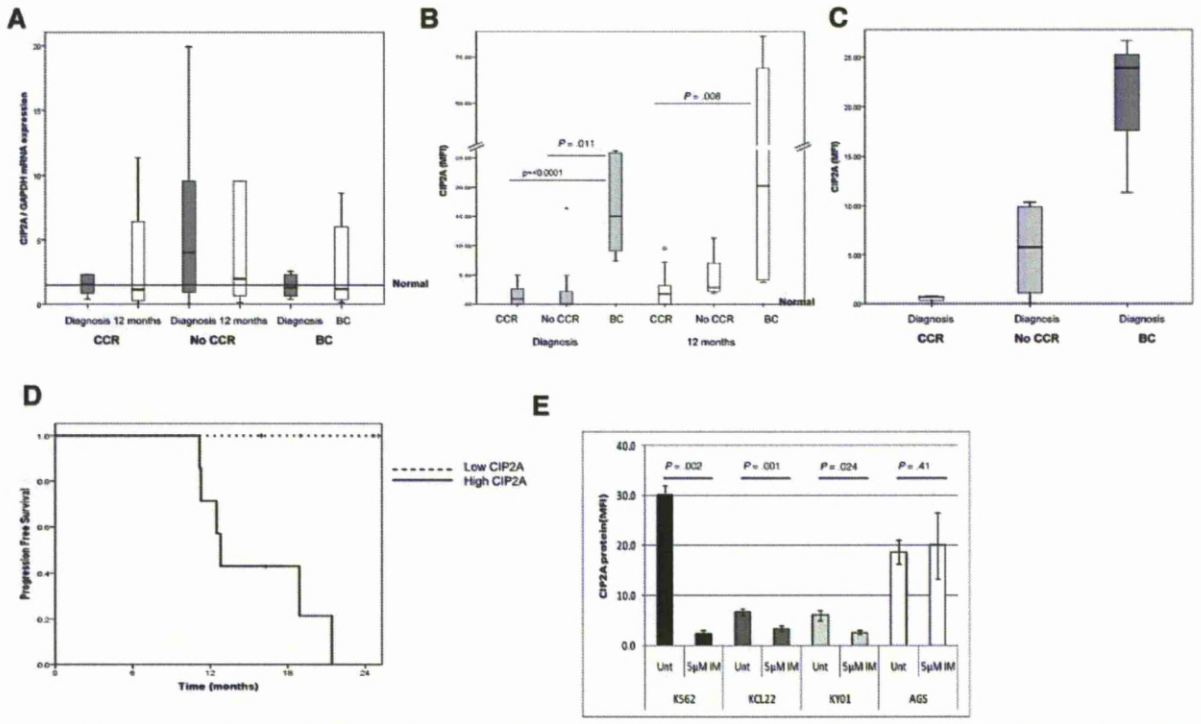


Figure 3. CIP2A protein level in MNCs and CD34⁺ at diagnosis is predictive of BC. (A) mRNA expression of CIP2A. No statistically significant difference was observed between any of the 3 response groups. CCR, n = 9; No-CCR, n = 9; and BC, n = 6. (B) Levels of CIP2A protein (mean level in 10 healthy subjects = 0.4; range, 0.17-0.63). CIP2A protein levels at diagnosis were significantly higher at diagnosis in patients who later progressed to BC than in CCR or No-CCR patients ($P < .0001$ and $P = .01$, respectively). As a patient progresses into BC, the CIP2A protein level increases further ($P = .008$). CCR, n = 11; No-CCR, n = 9; and BC, n = 6. (C) Level of CIP2A protein in diagnostic CD34⁺ cells stratified by the patients' clinical outcome. CIP2A is elevated in CD34 from patients destined to progress into BC. CCR, n = 3; No-CCR, n = 4; and BC, n = 3. (D) Kaplan-Meier plot of disease progression, stratified by CIP2A level at diagnosis. Patients with a high diagnostic CIP2A protein level (mean fluorescence intensity [MFI] > 5) have 100% probability of progressing to BC by 21 months ($P < .0001$). (E) CIP2A levels (mean ± SEM) in CML cell lines K562, KCL22, KY01, and a CIP2A-positive gastric cancer cell line AGS, before and after treatment with 5μM imatinib for 24 hours (n = 6).

target of PP2A. Flow cytometry was used to measure PIM1 expression in CD34⁺ cells from diagnosis. PIM1 was elevated in patients destined to progress into BC compared with the CCR and No-CCR patients (Figure 4C). In addition, analysis undertaken in K562 cells revealed that activation of PP2A, either by addition of a PP2A activator or inhibition of CIP2A by imatinib, also decreased PIM1 levels (data not shown). Considering the role of PP2A in regulating PIM1 protein levels, these data provide further evidence that PP2A activity is suppressed in the CD34⁺ cells of patients at high risk of developing BC.

Finally, previous work by others has indicated that suppression of PP2A activity feeds back on BCR-ABL1 to facilitate increased and sustained kinase activity.[8] One of the hallmarks of BCR-ABL1 activity is phosphorylation of CrkL,[3,27] and we have previously shown that an increased pCrkL/CrkL ratio is indicative of a poor response to imatinib.[3] We assessed the pCrkL/CrkL ratio on all

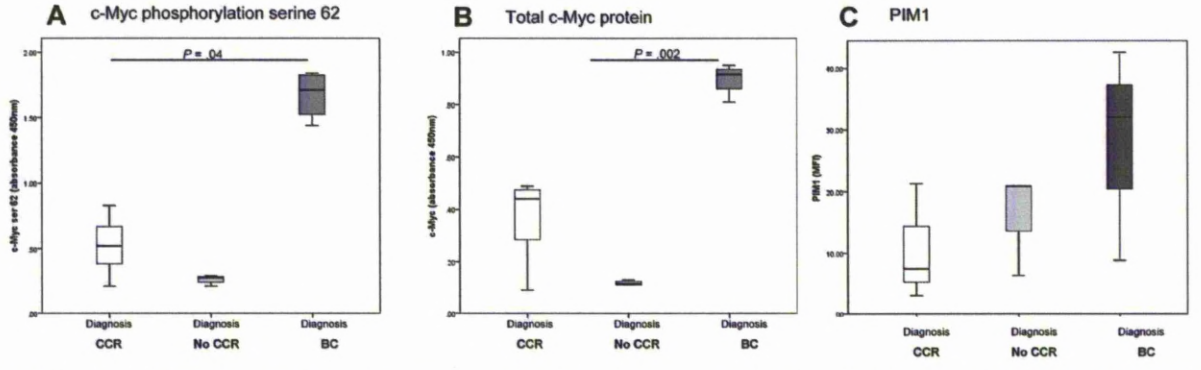


Figure 4. Assessing PP2A function. c-Myc is elevated at diagnosis in patients destined to progress into BC. Levels of c-Myc phosphorylation on serine 62 (A) and total c-Myc protein levels (B). Stratification by clinical outcome and abbreviations as for the previous figures. CCR, n = 10; No-CCR, n = 5; and BC, n = 5. (C) PIM1 is higher in the CD34⁺ cells from patients who will later progress into BC, confirming that PP2A function is inhibited. CCR, n = 3; No-CCR, n = 3; and BC, n = 3.

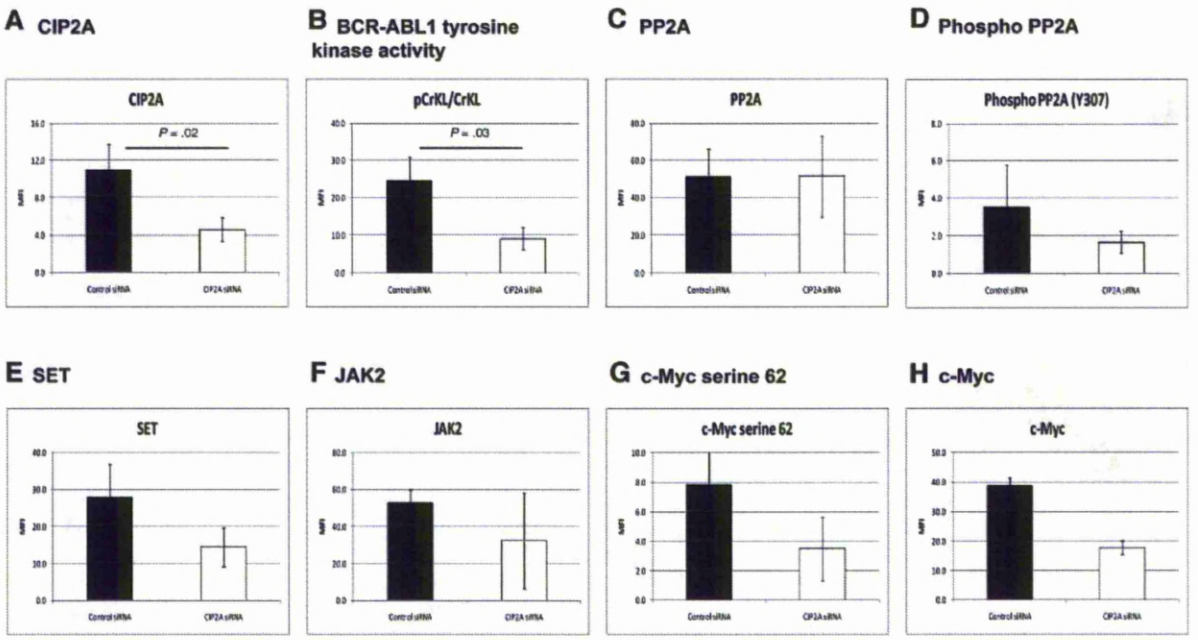


Figure 5. CIP2A plays a key role in regulating PP2A in CML cells. K562 cells were treated with CIP2A siRNA for 72 hours (n = 5). The effects are shown on: CIP2A protein levels as a control (A), BCR-ABL1 activity as assessed by the pCrkL/CrkL ratio (B), PP2A and phosphorylated PP2A (C-D), SET (E), and JAK2 (F). c-Myc phosphorylation at serine 62 and total c-Myc protein (G-H).

(n = 10) available samples from the present cohort of patients. BCR-ABL1 activity was highest in patients destined to progress into BC (supplemental Figure 4).

CIP2A plays a key role in regulating PP2A in CML cells

To confirm a role for PP2A in regulating c-Myc and PIM1 expression, the CML cell line K562 was treated with imatinib. Figure 3E demonstrates that this reduces CIP2A protein expression. pS^{62}-Myc and PIM1 levels were also reduced in the cells treated with imatinib (data not shown). These results are compatible with the notion that CIP2A expression regulated by BCR-ABL1 activity has a role in suppression of PP2A activity and in downstream activation of PIM1 and c-Myc.

To further investigate the effect of CIP2A on CML cells, K562 cells were treated with siRNA targeted at CIP2A (n = 5; Figure 5). After siRNA treatment, the mean knockdown of the CIP2A protein was 60% (P = .02; Figure 5A). Inhibition of CIP2A resulted in a 63% decrease in BCR-ABL1 tyrosine kinase activity as assessed by the pCrkL/CrkL ratio as previously described[3] (P = .03; Figure 5B). PP2A protein levels remained unchanged (Figure 5C), but the degree of PP2A phosphorylation (at Y307, indicating inactivity) decreased by 53%, indicative of an increase in PP2A function (Figure 5D). In addition, SET and JAK2 decreased by 48% and 37%, respectively, with CIP2A siRNA treatment, indicating that CIP2A acts upstream of JAK2 and SET (Figure 5E-F). Phosphorylation of c-Myc at serine 62 and total c-Myc protein also decreased by approximately 50% (Figure 5G-H). Key results from these experiments were also confirmed by Western blotting, using an alternative siRNA sequence, and on the additional CML cell line LAMA84 (shown in supplemental Figure 5). These data confirm that PP2A is functionally inactivated by CIP2A and that decreasing the level of CIP2A protein removes the block on PP2A function and decreases BCR-ABL1 activity.

Discussion

Despite the dramatic effect of imatinib, CML remains a fatal disease for the proportion of patients who progress from chronic phase to BC. Recent data suggest that the second-generation TKI nilotinib decreases the rate of progression to BC in the first 12 months,[28] but it remains to be seen whether this benefit is maintained at later time points. Our previously published biomarker data on the pCrKL/CrKL ratio and BCR-ABL1 transcript type can only predict patients likely to achieve a CCR and cannot predict patients destined to progress to BC.[3,19] Various techniques have been used on diagnostic chronic phase samples to predict who will develop BC,[29] and a variety of novel genomic lesions have been identified at BC.[30] It is however not possible at diagnosis to reliably predict which patients will develop disease progression. This is in part because of a poor understanding of the factors facilitating development of the novel genomic lesions that lead to disease progression.

The present data suggest that CIP2A is an important determinant of future disease progression in CML. We show that high CIP2A protein levels are present in diagnostic MNCs and $CD34^+$ cells of patients destined to progress into BC, and after imatinib treatment CIP2A levels increase further. The probability of disease progression is 100% at 21 months in patients with high CIP2A protein. Interestingly, no correlation was observed between CIP2A expression and Sokal score, suggesting that the presence of high levels of this protein is an independent biomarker of BC. In particular, of the 6 patients in this study who progressed to BC, only 2 had high Sokal scores (Table 1), whereas in the CCR group, 4 of the 14 patients had high Sokal but all achieved a CCR.

CIP2A functions as a regulator of PP2A activity, and high expression of this protein is reported to suppress the phosphatase

activity of PP2A.[12] We used specific siRNA and imatinib to reduce CIP2A levels in K562 cells and show that this results in a decrease in expression of 2 important PP2A targets, c-Myc and PIM1. These proteins are important because PIM1 is a kinase that acts to phosphorylate c-Myc and stabilize its expression.[23,26] When PIM1 is dephosphorylated by PP2A, it becomes degraded within the proteasome.[26] Similarly, c-Myc is also degraded in the proteosome once it is dephosphorylated by PP2A.[12] The reductions in PIM1 and c-Myc (and concomitant reduction of pS^{62}-Myc) expression therefore suggest that PP2A activity increases. This notion is supported by our observation that pY^{307}-PP2A levels decreased without affecting PP2A protein levels in the treated cells. Phosphorylation of PP2A on Y^{307} deactivates this phosphatase, and removal of this phosphate by PP2A-mediated autodephosphorylation results in its reactivation.[6] Taken together, the results of these experiments therefore suggest a mechanistic connection between high CIP2A expression, PP2A phosphorylation, and suppression of activity (illustrated by high levels of pY^{307}-PP2A) and high levels of pS^{62}-Myc and of c-Myc and PIM1 protein.

In the clinical samples we analyzed, we found that high CIP2A protein levels corresponded with high levels of Y^{307} and S^{62} phosphorylation of PP2A and c-Myc, respectively. Moreover, the patient samples containing high levels of CIP2A also had high levels of c-Myc and PIM1. Taken together with the cell line findings, these data connect CIP2A to c-Myc and PIM1 in primary CML cells and suggest that CIP2A, c-Myc, and PIM1 could be prognostic biomarkers.

High levels of pS^{62}-Myc and c-Myc protein may have important consequences. c-Myc has a critical role in cell proliferation, and increased levels of this protein within cells promote entry into cell cycle. Thus, diagnostic chronic phase cells with high c-Myc probably have a greater proliferative potential. This notion is supported by studies of clinical gastric cancer biopsies in which aggressive (proliferative) disease is associated with high expression of CIP2A and c-Myc.[10] Deregulated cell division is known to result in increased DNA mismatch repair errors and in genomic instability.[31] This is also observed in CML because c-Myc may contribute to disease progression by promoting aneuploidy.[31-34]

Whether CIP2A-mediated inhibition of PP2A acts directly to block dephosphorylation of c-Myc or acts at a stage upstream of c-Myc remains to be determined. Junttila et al provide evidence suggesting that CIP2A binds directly to c-Myc and is recruited as a result of S^{62} phosphorylation.[12] However, we found that the reduction of CIP2A resulted in a decrease in BCR-ABL1 activity, and a reduction in kinase activity would result in a decrease in the signals that are necessary to induce stable expression of c-Myc.

In the present study, patients from the CCR group had high levels of SET protein in samples taken at diagnosis. At the 12-month sampling, SET protein levels in the MNC from these patients were restored to almost normal levels. This is consistent with the in vitro findings of Neviani et al,[8] which demonstrated that PP2A is inhibited by SET and that PP2A function can be restored by a reduction in SET levels caused by imatinib-induced inhibition of BCR-ABL1 and JAK2. Broadly similar data were seen in the No-CCR group, who also did not undergo disease progression. However, patients in the BC group had lower SET levels than did patients who do not progress. This suggests that PP2A activity may be regulated differently in cells of CCR and BC patients; in the former group of patients, PP2A activity is regulated predominantly by SET, whereas in the latter group, PP2A activity is regulated by SET and CIP2A. Figure 6 summarizes the present findings and postulates possible mechanisms of how PP2A is inhibited in CML.

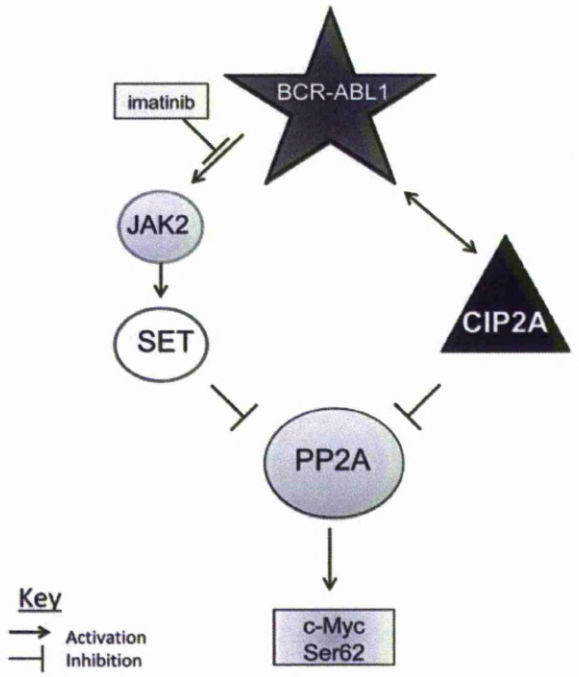


Figure 6. Model mechanisms by which CIP2A regulates PP2A and BCR-ABL1 signaling proteins. Possible mechanism of PP2A inhibition in CML. In most patients, PP2A is suppressed by SET, which is driven by BCR-ABL1 via JAK2. In patients destined to progress into BC, PP2A is inhibited by CIP2A both directly as well as through BCR-ABL.[1]

In patients who respond to imatinib therapy, PP2A activity in the malignant cells is suppressed by SET, and SET expression is regulated by BCR-ABL1 via JAK2.[8] When imatinib is commenced, BCR-ABL1 activity decreases and this leads to removal of PP2A inhibition through a decrease in SET protein levels. However, in patients who progress to BC, CIP2A is present at a high level and contributes to PP2A suppression. Imatinib therapy does not affect CIP2A levels in the malignant cells of these patients and PP2A remains suppressed. c-Myc expression therefore remains at a higher level, increasing the probability of subsequent genetic damage through increased cell proliferation and thus disease progression to BC.

In conclusion, the present data demonstrate that CIP2A is an important determinant of future disease progression, and its use as a biomarker of BC needs to be tested both in animal models and prospectively in clinical samples. The data also suggest that CIP2A may be a useful therapeutic target in CML.

Acknowledgments

The authors thank Alison Holcroft and Joanna Middleton of the University of Liverpool Biobank for their help and support during this project as well as Dr Mark Glenn for advising on the siRNA work.

Authorship

Contribution: C.M.L., R.J.H., and R.E.C. designed the study; C.M.L., R.J.H., J.R.S., and R.E.C. wrote the manuscript; C.M.L.

and A.G. performed the laboratory work for this study; and M.C. provided clinical samples.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Richard E. Clark, Department of Haematology, University of Liverpool, Liverpool, L69 3GA, United Kingdom; e-mail: clarkre@liv.ac.uk.

References

1. Lucas CM, Wang L, Austin GM, et al. A population study of imatinib in chronic myeloid leukaemia demonstrates lower efficacy than in clinical trials. *Leukemia.* 2008;22(10):1963-1966.
2. de Lavallade H, Apperley JF, Khorashad JS, et al. Imatinib for newly diagnosed patients with chronic myeloid leukemia: incidence of sustained responses in an intention-to-treat analysis. *J Clin Oncol.* 2008;26(20):3358-3363.
3. Lucas CM, Harris RJ, Giannoudis A, Knight K, Watmough SJ, Clark RE. BCR-ABL1 tyrosine kinase activity at diagnosis, as determined via the pCrkL/CrkL ratio, is predictive of clinical outcome in chronic myeloid leukaemia. *Br J Haematol.* 2010;149(3):458-460.
4. Sattler M, Salgia R, Okuda K, et al. The proto-oncogene product p120CBL and the adaptor proteins CRKL and c-CRK link c-ABL, p190BCR/ABL and p210BCR/ABL to the phosphatidylinositol-3' kinase pathway. *Oncogene.* 1996;12(4):839-846.
5. Sánchez-Arévalo Lobo VJ, Aceves Luquero CI, Alvarez-Vallina L, et al. Modulation of the p38 MAPK (mitogen-activated protein kinase) pathway through Bcr/Abl: implications in the cellular response to Ara-C. *Biochem J.* 2005;387(1):231-238.
6. Janssens V, Goris J. Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *Biochem J.* 2001;353(3):417-439.
7. Perrotti D, Neviani P. ReSETting PP2A tumour suppressor activity in blast crisis and imatinib-resistant chronic myelogenous leukaemia. *Br J Cancer.* 2006;95(7):775-781.
8. Neviani P, Santhanam R, Trotta R, et al. The tumor suppressor PP2A is functionally inactivated in blast crisis CML through the inhibitory activity of the BCR/ABL-regulated SET protein. *Cancer Cell.* 2005;8(5):355-368.
9. Samanta AK, Chakraborty SN, Wang Y, et al. Jak2 inhibition deactivates Lyn kinase through the SET-PP2A-SHP1 pathway, causing apoptosis in drug-resistant cells from chronic myelogenous leukemia patients. *Oncogene.* 2009;28(14):1669-1681.
10. Cristobal I, Blanco FJ, Garcia-Orti L, et al. SETBP1 overexpression is a novel leukemogenic mechanism that predicts adverse outcome in elderly patients with acute myeloid leukemia. *Blood.* 2010;115(3):615-625.
11. Lucas CM, Harris RJ, Giannoudis A, Davies A, Clark RE. SET binding protein 1 expression does not predict clinical outcome in chronic myeloid leukemia [e-letter]. *Blood.* 2010. http://bloodjournal.hematologylibrary.org/content/115/3/615.short/reply bloodjournal_el_59. Published April 1, 2010.
12. Junttila MR, Puustinen P, Niemela M, et al. CIP2A inhibits PP2A in human malignancies. *Cell.* 2007; 130(1):51-62.
13. Côme C, Laine A, Chanrion M, et al. CIP2A is associated with human breast cancer aggressivity. *Clin Cancer Res.* 2009;15(16):5092-5100.
14. Li W, Ge Z, Liu C, et al. CIP2A is overexpressed in gastric cancer and its depletion leads to impaired clonogenicity, senescence, or differentiation of tumor cells. *Clin Cancer Res.* 2008;14(12): 3722-3728.
15. Khanna A, Bockelman C, Hemmes A, et al. MYC-dependent regulation and prognostic role of CIP2A in gastric cancer. *J Natl Cancer Inst.* 2009; 101(11):793-805.
16. Kerosuo L, Fox H, Perälä N, et al. CIP2A increases self-renewal and is linked to Myc in neural progenitor cells. *Differentiation.* 2010;80(1): 68-77.
17. Wang J, Li W, Li L, Yu X, Jia J. CIP2A is overexpressed in acute myeloid leukaemia and associated with HL60 cells proliferation and differentiation. *Int J Lab Hematol.* 2011;33(3):290-298.
18. Coenen EA, Zwaan CM, Meyer C, et al. KIAA1524: a novel MLL translocation partner in acute myeloid leukemia. *Leuk Res.* 2011;35(1): 133-135.
19. Lucas CM, Harris RJ, Giannoudis A, et al. Chronic myeloid leukaemia patients with the e13a2 BCR-ABL fusion transcript have inferior responses to imatinib than e14a2 patients. *Haematologica.* 2009;94(10):1362-1367.
20. Wang L, Pearson K, Pillitteri L, Ferguson JE, Clark RE. Serial monitoring of BCR-ABL by peripheral blood real-time polymerase chain reaction predicts the marrow cytogenetic response to imatinib mesylate in chronic myeloid leukaemia. *Br J Haematol.* 2002;118(3):771-777.
21. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-[delta][delta]CT method. *Methods.* 2001;25(4):402-408.
22. Hamilton A, Alhashimi F, Myssina S, Jorgensen HG, Holyoake TL. Optimization of methods for the detection of BCR-ABL activity in Philadelphia-positive cells. *Exp Hematol.* 2009;37(3):395-401.
23. Wang J, Kim J, Roh M, et al. Pim1 kinase synergizes with c-MYC to induce advanced prostate carcinoma. *Oncogene.* 2010;29(17):2477-2487.
24. Chen J, Martin BL, Brautigan DL. Regulation of protein serine-threonine phosphatase type-2A by tyrosine phosphorylation. *Science.* 1992; 257(5074):1261-1264.
25. Brault L, Gasser C, Bracher F, Huber K, Knapp S, Schwaller J. PIM serine/threonine kinases in the pathogenesis and therapy of hematologic malignancies and solid cancers. *Haematologica.* 2010; 95(6):1004-1015.
26. Ma J, Arnold HK, Lilly M, Sears R, Kraft A. Negative regulation of Pim-1 protein kinase levels by the B56[beta] subunit of PP2A. *Oncogene.* 2007; 26(35):5145-5153.
27. White D, Saunders V, Lyons AB, et al. In vitro sensitivity to imatinib-induced inhibition of ABL kinase activity is predictive of molecular response in patients with de novo CML. *Blood.* 2005; 106(7):2520-2526.
28. Saglio G, Kim D-W, Issaragrisil S, et al. Nilotinib versus imatinib for newly diagnosed chronic myeloid leukemia. *N Engl J Med.* 2010;362(24): 2251-2259.
29. Yong ASM, Melo JV. The impact of gene profiling in chronic myeloid leukaemia. *Best Pract Res Clin Haematol.* 2009;22(2):181-190.
30. Calabretta B, Perrotti D. The biology of CML blast crisis. *Blood.* 2004;103(11):4010-4022.
31. Prochownik EV Li Y. The ever expanding role for c-Myc in promoting genomic instability. *Cell Cycle.* 2007;6(9):1024-1029.
32. Blick M, Romero P, Talpaz M, et al. Molecular characteristics of chronic myelogenous leukemia in blast crisis. *Cancer Genet Cytogenet.* 1987; 27(2):349-356.
33. McCarthy DM, Rassool FV, Goldman JM, Graham SV, Birnie GD. Genomic alterations involving the c-myc proto-oncogene locus during the evolution of a case of chronic granulocytic leukaemia. *Lancet.* 1984;2(8416):1362-1365.
34. Oudat R, Khan Z, Glassman AB. Detection of trisomy 8 in Philadelphia chromosome-positive CML patients using conventional cytogenetic and interphase fluorescence in situ hybridization techniques and its relation to c-myc involvement. *Ann Clin Lab Sci.* 2001;31(1):68-74.

Rapid diagnosis of chronic myeloid leukemia by flow cytometric detection of BCR-ABL1 protein

A definitive diagnosis of CML requires either the demonstration of the t(9;22) Philadelphia chromosome translocation (by FISH), or *BCR-ABL1* fusion gene (qRT-PCR). Both techniques provide important clinical information, but are usually confined to specialist regional laboratories as they are costly, require specialist staff to produce and interpret, and take several days to complete. Since most patients with neutrophilia and/or thrombocytosis do not have CML, a rapid and simple screening test for BCR-ABL1 protein would be clinically useful. Hitherto, demonstrating the BCR-ABL1 protein has been technically difficult because of its instability,[1] and the lack of a specific antibody that can reliably distinguish BCR-ABL1 protein from its normal ABL counterpart.

The BCR-ABL1 Protein kit (BD Biosciences) has been developed to detect all frequently occurring BCR-ABL1 fusion variants in human blood, including p190, p210 and p230. It uses an antibody recognizing BCR attached to a capture bead and an ABL-directed phycoerythrin (PE)–conjugated detection antibody. The presence of BCR-ABL1 protein results in a sandwich complex comprised of both the capture bead and the detection fluorophore, detectable by flow cytometry.

In initial optimization experiments on clinical samples, it was observed that using too few cells may result in a false negative result; thus a minimum of $1x10^7$ cells is recommended (Figure 1A). We compared BCR-ABL1 protein detection from total leukocytes or from MNC (Figure 1B). The BCR-ABL1 MFI (mean fluorescence intensity) signal was greater in MNC than total leukocytes (P=0.014). This may be because the proteolytic activity present within mature neutrophils is reduced within the MNC cell preparation. Comparing results from fresh or frozen MNC, whereby analysis was performed immediately after thawing frozen samples, we found no significant difference in detectable BCR-ABL1 protein. However, if the thawed cells were permitted to recover overnight prior to analysis, false negative results were seen in 2 out of 5 cases tested. The difference may be due to protease activity released from neutrophil granules disrupted by cell thawing.

BCR-ABL1 protein is known to be particularly sensitive to degradation within primary cells,[1] a feature that is not found in cell lines. It is, therefore, important to include a known BCR-ABL1 positive patient sample as a positive control.

We analyzed 157 samples for both BCR-ABL1 protein and BCR-ABL1 mRNA by qRT-PCR within 24-48 h of the sample being taken. These consisted of 110 samples from patients presenting with neutrophilia and/or thrombocytosis suspected of having CML, and 47 samples from normal healthy controls the data concerning which were kept anonymous. In all samples, the BCR-ABL1 status as determined by qRT-PCR was confirmed by the BCR-ABL1 protein assay with 100% concordance. The average MFI values from normal cellular samples were 87.7±5.5 giving a cut-off value between positives and negatives set at 99 (87.7±2SD) (Figure 2A).

Assay specificity and sensitivity were confirmed in primary cells by assaying for BCR-ABL1 protein in patients who had commenced treatment with a TKI. In patients who were in complete cytogenetic response (CCR, defined either by standard metaphase analysis on bone marrow or as a BCR-ABL1 qRT-PCR <1%)[2] the detectable BCR-ABL1 protein was either extremely low or undetectable. However, in patients at early stages of treatment but not yet in CCR (defined as a BCR-ABL1 ratio of 1-10%) BCR-ABL1 protein was readily detected (Figure 2A). In patients with newly diagnosed CML (BCR-ABL1 ratio >10%) BCR-ABL1 protein was extremely high (P=<0.0001).

The degree of decrease in BCR-ABL1 transcript level within the first three months of imatinib treatment can predict the subsequent clinical outcome.[3] To assess if a change in BCR-ABL1 protein level could also be predictive of later clinical outcome, fresh MNC taken at original diagnosis were cultured in the presence of imatinib for 24 h. The change in BCR-ABL1 protein level did not correlate with achievement of CCR. Furthermore, monitoring of the BCR-ABL1 protein level once treatment had commenced provided no useful clinical information (*data not shown*).

Use of any screening kit in a clinical diagnostic setting must be able to reliably detect all BCR-ABL1 transcript types. BCR-ABL1 protein was detected in all transcript types tested (e1a2, e13a2, e14a2 and both e13a2 and e14a2) with no significant differences in signal intensity between BCR-ABL1 transcript types (*data not shown*).

The BCR-ABL1 protein assay produces a test result from lysed cellular material in 4 h. However, access to CML primary cells as positive controls are required in order to eliminate false negative reporting, and these samples may not be readily available outside specialist laboratories. To deal with this problem, we have simplified the methodology by using a plasma based assay which eliminates confounding cellular granulocytic

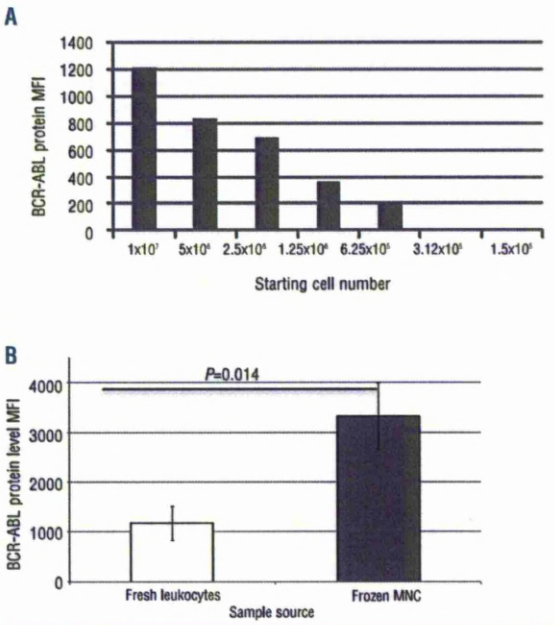


Figure 1. Optimization of sample source for the detection of BCR-ABL1 protein. (A) Assessing the number of cells required to detect BCR-ABL1 protein. (B) Comparison between fresh total leukocytes and frozen MNC.

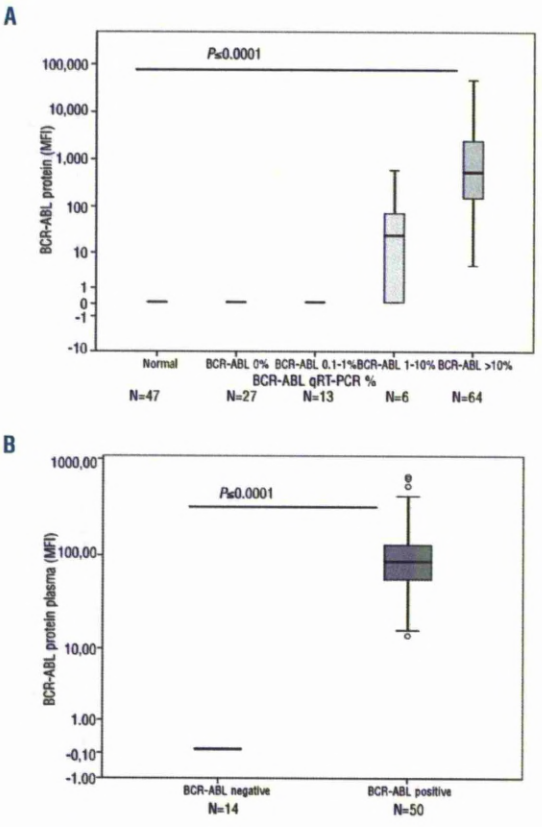


Figure 2. Screening clinical samples for BCR-ABL1 protein. (A) 157 cellular samples were screened for the presence of BCR-ABL1 protein; results are stratified by the BCR-ABL qRT-PCR results for the same sample. The MFI for each sample was determined as the observed MFI, minus the MFI value of the negative control plus two standard deviations. (B) 64 plasma samples were screened for the presence of the BCR-ABL1 protein. Fourteen from patients with neutrophilia and/or thrombocytosis or healthy controls normal plasma and 50 CML patients at diagnosis were studied.

degradation effects. It has previously been shown that leukemic cells shed their proteins, DNA and RNA into plasma.[4-6] Plasma was incubated with the capture beads and detector reagent for 6 h then analyzed for the presence of BCR-ABL1 protein.

Plasma samples were analyzed from 50 newly diagnosed CML patients and from 14 patients with neutrophilia and/or thrombocytosis or healthy controls. The normal plasma sample cut-off value was determined as 98 (72.4±2SD) MFI. In all cases, and for all transcript types tested, samples that were negative by plasma were also negative by cell lysate, and the result was always in agreement with the prevailing BCR-ABL1 mRNA level as defined by qRT-PCR (Figure 2B). These data demonstrate that the simplified plasma assay is also 100% accurate at detecting BCR-ABL1 protein. The level of BCR-ABL1 protein in a plasma sample did not correlate with the sample white blood count (*data not shown*).

In conclusion, when screening patients presenting with high white counts for CML, it is important to have a technique which economises on time and cost, since most patients will be negative and will not require further detailed testing. Furthermore, a rapid and reliable screening test will give rise to earlier diagnosis and clinical intervention. The present data show that this assay is sufficiently reliable to provide a first-line screening test for CML diagnosis. Applying this to a plasma sample rather than a cell lysate may provide further simplification, and this may be particularly useful in developing countries whose hospitals lack definitive facilities for the detection of BCR-ABL1.

Claire M. Lucas, Jemma L. Fagan, Anthony Carter, Bryony Swale, Craig Evans, Richard E. Clark, and Robert J. Harris

Department of Haematology, University of Liverpool, Liverpool, UK

Correspondence: Claire M. Lucas, Department of Haematology, Royal Liverpool University Hospital, Prescot Street, Liverpool L7 8XP, UK. Phone/Fax: international + 0151.7064344. E-mail: cml@liv.ac.uk

Key words: CML, diagnosis, flow cytometry, BCR-ABL1 protein.

Acknowledgments: we would like to thank the University of Liverpool Biobank staff Alison Holcroft and Joanna Middleton for their help and support during this project.

Citation: Lucas CM, Fagan JL, Carter A, Swale B, Evans C, Clark RE, and Harris RJ. Rapid diagnosis of chronic myeloid leukemia by flow cytometric detection of BCR-ABL1 protein Haematologica 2011; 96(07):1077-1078. doi:10.3324/haematol.2011.043166

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

Financial and other disclosures provided by the authors using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.

References

1. Patel H, Marley SB, Gordon MY. Conventional Western blotting techniques will not reliably quantify p210BCR-ABL1 levels in CML mononuclear cells. Blood. 2007;109(3):1335.
2. Wang L, Pearson K, Pillitteri L, Ferguson JE, Clark RE. Serial monitoring of BCR-ABL by peripheral blood real-time polymerase chain reaction predicts the marrow cytogenetic response to imatinib mesylate in chronic myeloid leukaemia. Br J Haematol. 2002;118(3):771-7.
3. Wang L, Pearson K, Ferguson JE, Clark RE. The early molecular response to imatinib predicts cytogenetic and clinical outcome in chronic myeloid leukaemia. Br J Haematol. 2003;120(6):990-9.
4. Rogers A, Joe Y, Manshouri T, Dey A, Jilani I, Giles F, et al. Relative increase in leukemia-specific DNA in peripheral blood plasma from patients with acute myeloid leukemia and myelodysplasia. Blood. 2004;103(7):2799-801.
5. Albitar M, Do K-A, Johnson MM, Giles FJ, Jilani I, O'Brien S, et al. Free circulating soluble CD52 as a tumor marker in chronic lymphocytic leukemia and its implication in therapy with anti-CD52 antibodies. Cancer. 2004;101(5):999-1008.
6. Jilani I, Kantarjian H, Faraji H, Gorre M, Cortes J, Ottmann O, et al. An immunological method for the detection of BCR-ABL fusion protein and monitoring its activation. Leukemia Research. 2008;32(6):936-43.

SET Binding Protein 1 expression does not predict clinical outcome in chronic myeloid leukaemia

Claire M Lucas, Scientist Robert J Harris, Athina Giannoudis, Andrea Davies and Richard E Clark.

Department of Haematology, Royal Liverpool University Hospital. Liverpool, England L7 8XP

To the Editor,

Recently Cristobal et al1 reported that overexpression of SETBP1, the binding protein for SET, predicts adverse outcome in acute myeloid leukaemia (AML). SETBP1 overexpression protects SET from protease cleavage, leading to increased amounts of full-length SET and formation of a SETBP1-SET-PP2A complex, which in turn results in inhibition of the tumour suppressor PP2A. Inhibition of PP2A provides abnormal proliferation and differentiation in transformed malignant cells. In chronic myeloid leukaemia (CML), BCR-ABL has been shown to induce the expression of SET, which inhibits PP2A activity.2 About one-third of CML patients will eventually fail imatinib treatment.3,4

We used the TaqMan gene expression assay system (Applied Biosystems) as used by Cristobal et al;1 cDNA extracted from peripheral blood was studied from 4 healthy subjects and 46 chronic phase CML patients treated with imatinib 400mg from diagnosis to investigate the relationship between SET and SETBP1 expression and clinical outcome. Their mean age was 48 years, with 22 males and 24 females. Sokal scores were as follows - low = 16; intermediate = 13; high = 16; unassigned = 1. Patients diagnostic samples were categorised according to their subsequent outcome at 12 months - 1) complete cytogenetic response (CCR), 2) no CCR and 3) those who progressed to blast crisis (BC).

SETBP1 expression was not statistically different between any groups (Table 1). However, SET expression was lower in the BC group compared to the CCR group (p= 0.04). No difference in SET expression was observed between no CCR and BC group, or between CCR and no CCR groups. Furthermore, unlike AML, SETBP1 expression levels in CML samples were lower than normal subjects. In contrast to AML, we conclude that SETBP1 expression at diagnosis is not predictive of clinical outcome following imatinib treatment in CML. Other factors may therefore be of greater importance in the regulation of PP2A and SET in CML.

Table 1. SET and SETBP1 expression levels at diagnosis in 46 CML patients, stratified by clinical outcome following imatinib treatment. Samples normalised to normal healthy controls. SET expression was statistically significant between patients who achieve a CCR and those who subsequently progress to blast crisis (p=0.04 Mann-Whitney U test)

		SET mRNA expression		SETBP1 mRNA expression	
		Average	Range	Average	Range
Normal value	n=4	1		1	
CCR	n=20	0.98	(0.01-6.10)	0.22	(0.00-0.16)
No CCR	n=21	0.60	(0.01-3.41)	0.34	(0.01-1.95)
Blast crisis	n=5	0.18	(0.09-0.31)	0.13	(0.03-0.42)

References

1. Cristobal I, Blanco FJ, Garcia-Orti L, et al. SETBP1 overexpression is a novel leukemogenic mechanism that predicts adverse outcome in elderly patients with acute myeloid leukemia. Blood. 2010;115:615-625.

2. Neviani P, Santhanam R, Trotta R, et al. The tumor suppressor PP2A is functionally inactivated in blast crisis CML through the inhibitory activity of the BCR/ABL-regulated SET protein. Cancer Cell. 2005;8:355- 368.

3. Lucas CM, Wang L, Austin GM, et al. A population study of imatinib in chronic myeloid leukaemia demonstrates lower efficacy than in clinical trials. Leukemia. 2008;22:1963-1966.

4. de Lavallade H, Apperley JF, Khorashad JS, et al. Imatinib for Newly Diagnosed Patients With Chronic Myeloid Leukemia: Incidence of Sustained Responses in an Intention-to-Treat Analysis. Journal of Clinical Oncology. 2008;26:3358-3363.

Conflict of Interest:

None declared

... less

Submit response

Published April 1, 2010

Chronic myeloid leukemia patients with the e13a2 *BCR-ABL* fusion transcript have inferior responses to imatinib compared to patients with the e14a2 transcript

Claire M. Lucas, Robert J. Harris, Athina Giannoudis, Andrea Davies, Katy Knight, Sarah J. Watmough, Lihui Wang, and Richard E. Clark

Department of Haematology, Royal Liverpool University Hospital, Liverpool, UK

ABSTRACT

Background

Chronic myeloid leukemia is characterized by a reciprocal translocation between chromosomes 9 and 22, creating the fusion gene *BCR-ABL*. The clinical significance of the type of *BCR-ABL* transcript in newly diagnosed patients in chronic phase treated with imatinib 400 mg from initial diagnosis remains unknown.

Design and Methods

We analyzed the clinical outcome of 78 newly diagnosed chronic phase patients, aged 16 or over, treated with imatinib 400 mg. Of these, 71 expressed either e13a2 or e14a2 transcripts. *BCR-ABL* transcripts were assayed by quantitative real-time polymerase chain reaction.

Results

After 12 months of treatment, 54% of the e14a2 patients had achieved a complete cytogenetic response, compared to 25% of the e13a2 patients (p=0.01). Kaplan-Meier analysis of the time to achieve complete cytogenetic response revealed that e14a2 patients had more rapid response rates, compared to e13a2 patients (p=0.006). e14a2 patients had a higher event-free survival rate in the first 12 months of treatment, although overall survival did not differ significantly between the patients with the two types of transcript. Human organic cation transporter protein 1 mRNA levels did not differ between the patients with the two types of transcript. The pre-treatment pCrKL/CrKL ratio (a surrogate marker of BCR-ABL tyrosine kinase activity) was higher in patients with e13a2 transcripts than in those with e14a2 (p=0.017).

Conclusions

Patients expressing the e14a2 transcript type have a higher rate and more rapid complete cytogenetic responses than e13a2-expressing patients, which may be due to higher BCR-ABL tyrosine kinase activity. Knowledge of the transcript type may yield additional prognostic information, although this requires testing on larger datasets.

Key words: chronic myeloid leukemia, BCR-ABL fusion transcript, imatinib.

Citation: Lucas CM, Harris RJ, Giannoudis A, Davies A, Knight K, Watmough SJ, Wang L, and Clark RE. Chronic myeloid leukemia patients with the e13a2 BCR-ABL *fusion transcript have inferior responses to imatinib compared to patients with the e14a2 transcript. Haematologica 2009;94:1362-1367. doi:10.3324/haematol.2009.009134*

Manuscript received on March 23, 2009. Manuscript accepted on May 6, 2009.

Correspondence:
Richard Clark,
Department of Haematology,
Royal Liverpool University Hospital, Prescot Street,
Liverpool, L7 8XP, UK.
E-mail: r.e.clark@liv.ac.uk.

Introduction

Chronic myeloid leukemia (CML) is characterized by a reciprocal translocation between chromosomes 9 and 22, which creates the fusion gene *BCR-ABL*. The breakpoints of chromosome 22 cluster within a small (5.8 kb) region, spanning exons e12-16 (formally denoted as b1-5) known as the major breakpoint cluster region (M-BCR). Breakpoint locations almost always fall between either exons e13 and e14 or between e14 and e15. Although the breakpoints in the *ABL* gene are also variable, because of splicing events, the transcribed mRNA has either an e13a2 (b2a2) or an e14a2 (b3a2) junction. The e13a2 and e14a2 *BCR-ABL* transcripts differ in length by 75 bp (25 amino acids).[1] Both *BCR-ABL* mRNA molecules encode a 210 kDa constitutively active protein kinase which is central to the pathogenesis of the disease.[2]

Imatinib is a tyrosine kinase inhibitor which has become the treatment of choice for newly diagnosed patients in chronic phase CML, and a recent report suggests that modern drug treatment may produce superior results to allogeneic stem cell transplantation.[3] We have recently shown in a population-based study[4] that by 24 months 49% of patients will fail imatinib treatment; similar findings were recently reported in a single center study.[5] This suggests that the identification of prognostic markers predictive of treatment response may be useful in order to avoid delay in offering second-line treatment such as stem cell transplantation or second-generation tyrosine kinase inhibitors.

Previous studies prior to the introduction of imatinib did not, in general, identify an effect of *BCR-ABL* transcript type on clinical outcome.[6-12] In the imatinib era one small study of 22 patients in different phases of disease suggested that patients with the e13a2 *BCR-ABL* transcript may be more sensitive to imatinib treatment;[13] while a larger study indicated that patients with e14a2 have a better molecular response to imatinib.[14] The clinical significance of the type of *BCR-ABL* transcript in newly diagnosed chronic phase CML patients treated with imatinib remains uncertain. Here we present the results of a population-based study in a single contiguous geographical locality investigating the effects of *BCR-ABL* transcript type on clinical outcome in 78 newly diagnosed patients with chronic phase CML treated with imatinib 400 mg.

Design and Methods

Patients and collection of samples

In our area of the north-west of England, the adjacent coastal strip of North Wales and the Isle of Man (total population 2 million), all services for adults with hematologic cancers are located in 12 hospitals. The molecular diagnosis of CML and monitoring for *BCR-ABL* transcripts for all CML patients in this geographical area are carried out in a single center (Royal Liverpool University Hospital). We are, therefore, able to trace the clinical course of every CML patient in our area. Peripheral blood samples were routinely collected at 3-monthly intervals for molecular monitoring. Briefly RNA was isolated from total white blood cells, cDNA was prepared and *BCR-ABL* transcripts were measured by real-time quantitative polymerase chain reaction (PCR), using a LightCycler as previously decribed.[15]

All 78 patients aged 16 or over with chronic phase CML newly diagnosed between January 1st 2003 and October 31st 2007 and with a minimum of 12 months follow-up were included in this study. The 71 patients who presented with either e13a2 or e14a2 *BCR-ABL* transcripts are the subject of the main investigation; patients presenting with both e13a2 and e14a2 transcripts are discussed separately (n=3). Patients expressing rare transcript types were excluded from this study (n=4; one each with e1a2, e14a3, e13a3 and one patient who expressed both e14a2 and e1a2 transcripts). Patients were included in the assessment of *BCR-ABL* transcript type if they received imatinib 400 mg daily from original diagnosis (preceded only by up to 6 weeks of hydroxycarbamide).

Measurement of CrKL phosphorylation by fluorescence-activated cell sorting

Phosphorylation of the CT10 regulator of kinase-like adaptor protein (CrKL) was used as a measure of BCR-ABL tyrosine kinase activity.[16] Cells (~5×10^5) were resuspended in 500 µL of 2% paraformaldehyde (VWR, Lutterworth, UK) and fixed for 10 min at 37°C. Cells were then chilled on ice for 1 min and centrifuged at 770 g for 3 min. Next, 500 µL of 90% methanol (Fisher Scientific, Leicestershire, UK) were added to the cell pellet. The cells were vortexed and then incubated on ice for 30 min. Cells were then washed (throughout with 1 mL incubation buffer containing phosphate-buffered saline and 0.5% bovine serum albumin), and centrifuged at 770g for 3 min. Cells were resuspended in 25 µL of incubation buffer and left at room temperature for 10 min. pCrKL antibody (28 µg/mL; Cell Signaling Technology, Massachusetts, USA) or CrKL antibody (28 µg/mL; Santa Cruz Biotechnology, California, USA) was added and 28 µg/mL anti-normal-rabbit immunoglobulins (R&D Systems, Abingdon, UK) used as a control. Cells were vortexed and incubated at room temperature for 40 before being washed twice and resuspended in 100 µL of incubation buffer containing 10 µg/mL fluorescein-labeled goat anti-rabbit second antibody Alexa Fluor 488 (Invitrogen, Paisley, UK), incubated at room temperature in the dark for 30 min, then washed twice and analyzed using flow cytometry (FACScalibur; Becton Dickinson, Oxford, UK), with Cellquest Pro software (Becton Dickinson) for data analysis. The pCrKL/CrKL ratio of a sample was determined using the following equation:

$$\text{pCrKL/CrKL ratio} = \frac{\text{pCrKL} - \text{control}}{\text{CrKL} - \text{control}} \times 100$$

Human organic cation transporter protein 1 mRNA analysis

Levels of human organic cation transporter protein 1 (hOCT1, SLC22A1) were determined using pre-treat-

ment cDNA, as previously described, on all suitable samples expressing either e13a2 or e14a2 transcripts.[17]

Clinical response

Responses were defined conventionally:[18] complete hematologic response was defined as normalization of the blood count and resolution of splenomegaly and complete cytogenetic response (CCR) was defined as no Philadelphia chromosome-positive metaphases among at least 20 bone marrow metaphases. In some cases serial cytogenetic data were not available and the achievement of CCR was determined by a *BCR-ABL/ABL* transcript ratio of less than 1%, which we have previously shown to be strongly correlated with cytogenetically defined CCR;[15] we, therefore, use the term *CCR equivalence* (CCRe) to encompass these cases.[4]

Statistical analysis

Statistical analysis and comparisons were performed using the statistical program SPSS 16.0 (SPSS Inc. Chicago, USA). Fishers' test and Mann-Whitney tests were used.

Results

During the study 78 patients with newly diagnosed chronic phase CML were assessed, of whom four expressed a rare/variant transcript type and were, therefore, excluded from this analysis. Three cases co-expressed e13a2 and e14a2 and for the purpose of this study they are considered as a separate group, leaving 71 cases expressing either e13a2 or e14a2 *BCR-ABL* transcripts. Of these, 32 expressed the e13a2 type of *BCR-ABL* transcript type and 39 expressed the e14a2 type of transcript. Age, sex and Sokal score of all cases analyzed are presented in Table 1.

Outcome according to transcript type

At 12 months 25% of patients with the e13a2 transcript had achieved a CCRe compared to 54% of e14a2 patients (p=0.01, Table 2). At 18 and 24 months the e13a2 patients continued to have lower rates of CCRe compared to those of the e14a2 patients. Kaplan-Meier analysis of time to achieve CCRe revealed that the patients with the e14a2 transcript type had more rapid responses than did the e13a2 patients (Figure 1A), with this effect continuing throughout treatment (p=0.006).

Patients with the e14a2 transcript type demonstrated a non-significant trend toward a higher event-free survival rate in the first 12 months of treatment, although overall survival rates did not differ significantly between patients with the two types of the transcript (Figure 1B and C). This trend is consistent with the finding that more patients with e13a2 progressed in the first 12 months of treatment; imatinib treatment failed in eight patients with e13a2 (7 had disease progression and 1 was intolerant to therapy), while treatment failed in only three patients with the e14a2 transcript (2 had disease progression and 1 was intolerant to therapy). The Sokal score was not predictive of clinical outcome nor did it differ according to the type of transcript (Table 1). Of interest, five additional cases outside this study who presented with blast crisis CML during the period of the study all expressed the e13a2 transcript type.

Additionally three cases presented with both e13a2 and e14a2 *BCR-ABL* transcripts at diagnosis. Following 12 months of treatment two achieved a CCRe, although by 24 months one of these patients had subsequently lost the CCRe. The patient who failed to

Table 1. (A) Summary of the patients' characteristics.

Number of patients	71	32	39
Age (years)	50 (19-81)	48 (19-75)	51 (19-81)
Sex (male/female)	36/35	19/13	17/22
Sokal score			
High	21	9	12
Intermediate	14	5	9
Low	15	6	9
No data	21	12	9

Table 1. (B) CCRe rates for both e13a2 and e14a2 *BCR-ABL* transcript types at 12, 18 and 24 months.*

	12 months			18 months			24 months		
	Total patients	CCRe	% of total patients achieving CCRe	Total patients	CCRe	% of total patients achieving CCRe	Total patients	CCRe	% of total patients achieving CCRe
Transcript type									
e13a2	32	8	25.0	23	8	34.8	23	9	39.1
e14a2	39	21	53.8	32	18	56.3	26	15	57.7

**At 18 months nine cases with e13a2 and seven cases with e14a2 are excluded from analysis as not yet having received 18 months of Imatinib treatment. At 24 months six further cases with e14a2 are excluded for the same reason.*

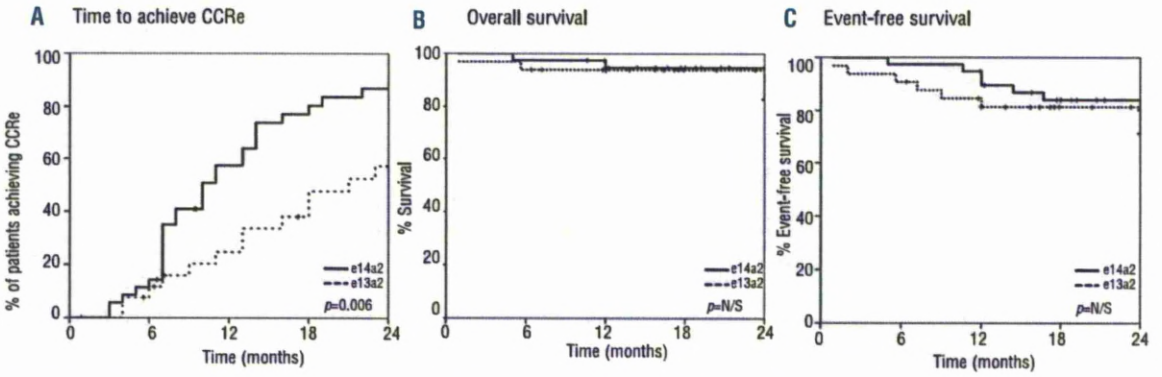


Figure 1. Kaplan Meier plots. (A) Kaplan Meier estimate of time to achieve CCRe for patients with the e13a2 and e14a2 *BCR-ABL* transcripts, demonstrating a slower response for e13a2 patients (p=0.006). (B) Overall survival stratified by *BCR-ABL* transcript type and (C) event-free survival; no statistically significant differences were observed (SPSS statistical package Mantel-Cox log-rank test).

achieve a CCRe following imatinib treatment was switched to an alternative tyrosine kinase inhibitor but failed to achieve a CCRe.

Correlation of transcript type with human organic cation transporter protein 1 and BCR-ABL kinase activity

Imatinib uptake into CML cells is dependent on hOCT1,[19] while the pCrKL/CrKL ratio is a surrogate marker for BCR-ABL tyrosine kinase activity.[16] In an attempt to establish why patients with the two different types of transcript respond differently to imatinib treatment we determined hOCT1 mRNA levels (n=51) and the pCrKL/CrKL ratio (n=28) in all patients for whom suitable material was available. No relationship was found between transcript type and hOCT1 mRNA (*data not shown*). However, samples from e13a2 patients had a higher pCrKL/CrKL ratio than those from e14a2 patients (p=0.017), demonstrating a higher tyrosine kinase activity (Figure 2).

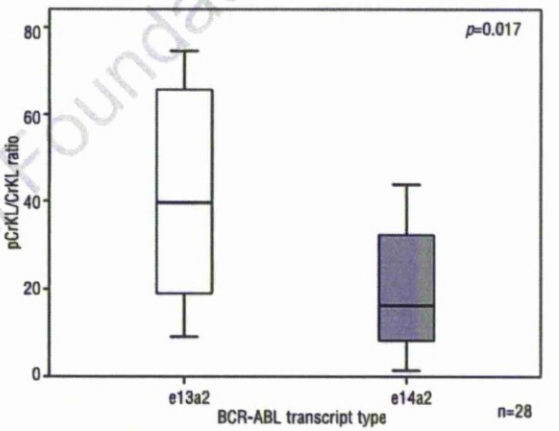


Figure 2. pCrKL/CrKL ratio for patients with the e13a2 and e14a2 transcripts pCrKL/CrKL ratio was measured in 28 newly diagnosed chronic CML patients prior to treatment as a surrogate marker for BCR-ABL tyrosine kinase activity, the pCrKL/CrKL ratio was higher in e13a2 patients than in e14a2 patients.

Discussion

In the pre-imatinib era, numerous studies addressed the significance of the *BCR-ABL* transcript type on CML outcome. Confusion can arise because some studies pooled e13a2 and e14a2 transcript types as 5' breakpoints; which were then compared to 3' breakpoints. These 3' breakpoints include e1a2 fusion transcripts that encodes a p190 protein, which is associated with acute lymphoblastic leukemia and has a poorer outcome than chronic phase CML. In general no difference in outcome was found in the pre-imatinib era between patients with chronic phase CML expressing e13a2 or e14a2 transcripts. However, surprisingly little information is available on the effects of these transcripts on response to imatinib treatment. In this study we focused on comparisons between outcomes in patients with e13a2 and e14a2 transcripts.

The e13a2 junction may be more prevalent in men,[20] although not necessarily in all ethnic groups, and has been associated with blast crisis of myeloid phenotype.[7] The e14a2 transcript has been correlated with a higher platelet count in both adults[8,10] and children.[21] Additionally it has been associated with a longer chronic phase and survival, possibly related to the less aggressive course of chronic phase in patients with this transcript type.[22] Furthermore, patients with the e14a2 transcript were found to have a higher level of *5'ABL* deletions as an additional chromosomal abnormality, when compared to e13a2-expressing patients.[7] Other studies found no correlations with the above, or with any other clinical or hematologic parameters.[20,22-24]

Overall, data from the pre-imatinib era suggest that transcript type has no influence on clinical outcome.[6-12] However, to our knowledge, only two studies have addressed the effect of transcript type on the clinical outcome of patients treated with imatinib. One small study of 22 patients in Brazil with variable disease status found a significant difference in response to treatment at 6 months (p=0.0347), with e13a2-expressing patients responding better than those expressing e14a2.[13] A much larger study of patients in chronic phase treated with imatinib concluded that patients with e14a2 had a higher probability of achieving a major molecular response, and had a greater reduction in overall transcript levels in response to imatinib than did e13a2-expressing patients.[14]

Here we present additional data on all the patients in our contiguous geographical area. These data support the view that patients with the e14a2 transcript may respond better to imatinib 400 mg daily. Following 12 months of imatinib treatment e14a2 patients not only achieved significantly higher CCRe rates than e13a2 patients but also achieved their CCRe at a faster rate. This was not related to the patients' age, sex or Sokal score which takes into consideration standard hematologic parameters.[25] Additionally hOCT1 expression did not differ between e13a2 and e14a2 patients; thus, the degree of imatinib uptake between patients with the two types of transcript is similar and does not account for the observed differences in clinical responses.

The reason for the difference in response to imatinib therapy therefore appears to be unrelated to differences in imatinib transport between transcript types. It is, however, likely that the effect is due to differences in the drug target. Using the pCrKL/CrKL ratio as assessment of BCR-ABL tyrosine kinase activity,[16] it was found that tyrosine kinase activity is higher in e13a2 patients than in patients with e14a2. A fixed dose of imatinib may, therefore, suppress the lower kinase activity in e14a2 patients to a greater proportional extent than the higher kinase levels in e13a2 patients. This may explain the higher incidence of CCRe following treatment with imatinib in e14a2 patients.

In conclusion, patients with the e14a2 *BCR-ABL* transcript type have a higher response rate to imatinib when the CCRe rate is compared to patients who express the e13a2 transcript type. We suggest that this maybe due to e13a2 patients having a higher BCR-ABL tyrosine kinase activity. Knowledge of patient transcript type may yield clinically useful data, and should be included in future clinical trials of tyrosine kinase inhibitors.

Authorship and Disclosures

CML, RJH and REC designed the study and wrote the manuscript; CML, AG, AD and LW performed the laboratory work for this study; KK and SW were responsible for taking patients' samples and providing clinical information; REC was the principal investigator.

The authors reported no potential conflicts of interest.

References

1. Deininger MW, Goldman JM, Melo JV. The molecular biology of chronic myeloid leukemia. Blood 2000;96: 3343-56.
2. Ben-Neriah Y, Daley GQ, Mes-Masson AM, Witte ON, Baltimore D. The chronic myelogenous leukemia-specific p210 protein is the product of the BCR/ABL hybrid gene. Science 1986;233:212-4.
3. Hehlmann R, Berger U, Pfirrmann M, Heimpel H, Hochhaus A, Hasford J, et al. Drug treatment is superior to allografting as first-line therapy in chronic myeloid leukemia. Blood 2007;109:4686-92.
4. Lucas CM, Wang L, Austin GM, Knight K, Watmough SJ, Shwe KH, et al. A population study of imatinib in chronic myeloid leukaemia demonstrates lower efficacy than in clinical trials. Leukemia 2008;22: 1963-6.
5. de Lavallade H, Apperley JF, Khorashad JS, Milojkovic D, Reid AG, Bua M, et al. Imatinib for newly diagnosed patients with chronic myeloid leukemia: incidence of sustained responses in an intention-to-treat analysis. J Clin Oncol 2008;26: 3358-63.
6. Morris SW, Daniel L, Ahmed CM, Elias A, Lebowitz P. Relationship of bcr breakpoint to chronic phase duration, survival, and blast crisis lineage in chronic myelogenous leukemia patients presenting in early chronic phase. Blood 1990;75:2035-41.
7. Polampalli SCN, Negi N, Shinde S, Baisane C, Amre P, Subramanian PG, et al. Analysis and comparison of clinicohematological parameters and molecular and cytogenetic response of two Bcr/Abl fusion transcripts. Gen Mol Res 2008;7:1138-49.
8. Rosas-Cabral A, Martínez-Mancilla M, Ayala-Sánchez M, Vela-Ojeda J, Bahena-Reséndiz P, Vadillo-Buenfil M, et al. Analysis of Bcr-abl type transcript and its relationship with platelet count in Mexican patients with chronic myeloid leukemia. Gaceta Méx 2003;139:553-9.
9. Meissner RDV, Dias PMB, Covas DT, Job F, Leite M, Nardi NB. A polymorphism in exon b2 of the major breakpoint cluster region (M-bcr) identified in chronic myeloid leukaemia patients. Br J Haematol 1998;103:224-6.
10. Perego RA, Costantini M, Cornacchini G, Gargantini L, Bianchi C, Pungolino E, et al. The possible influences of B2A2 and B3A2 BCR/ABL protein structure on thrombopoiesis in chronic myeloid leukaemia. Eur J Cancer 2000;36: 1395-401.
11. Shepherd P, Suffolk R, Halsey J, Allan N. Analysis of molecular breakpoint and m-RNA transcripts in a prospective randomized trial of interferon in chronic myeloid leukaemia: no correlation with clinical features, cytogenetic response, duration of chronic phase, or survival. Br J Haematol 1995;89:546-54.
12. Mills KI, Benn P, Birnie GD. Does the breakpoint within the major breakpoint cluster region (M-bcr) influence the duration of the chronic phase in chronic myeloid leukemia? An analytical comparison of current literature. Blood 1991;78:1155-61.
13. de Lemos JA, de Oliveira CM, Scerni AC, Bentes AQ, Beltrao AC, Bentes IR, et al. Differential molecular response of the transcripts B2A2 and B3A2 to imatinib mesylate in chronic myeloid leukemia. Gen Mol Res 2005;30:4:803-11.
14. Vega-Ruiz A, Kantarjian H, Shan J, Wierda W, Burger J, Verstovsek S, et al. Better molecular response to imatinib for patients (pts) with chronic myeloid leukemia (CML) in chronic phase (CP) carrying the b3a2 transcript compared to b2a2. ASH Annual Meeting Abstracts 2007;110: 1939.
15. Wang L, Pearson K, Pillitteri L, Ferguson JE, Clark RE. Serial moni-

toring of BCR-ABL by peripheral blood real-time polymerase chain reaction predicts the marrow cytogenetic response to imatinib mesylate in chronic myeloid leukaemia. Br J Haematol 2002;118:771-7.
16. White D, Saunders V, Lyons AB, Branford S, Grigg A, To LB, et al. In vitro sensitivity to imatinib-induced inhibition of ABL kinase activity is predictive of molecular response in patients with de novo CML. Blood 2005;106:2520-6.
17. Wang L, Giannoudis A, Lane S, Williamson P, Pirmohamed M, Clark RE. Expression of the uptake drug transporter hOCT1 is an important clinical determinant of the response to imatinib in chronic myeloid leukemia. Clin Pharmacol Ther 2007;83:258-64.
18. Baccarani M, Saglio G, Goldman J, Hochhaus A, Simonsson B, Appelbaum F, et al. Evolving concepts in the management of chronic myeloid leukemia: recommendations from an expert panel on behalf of the European LeukemiaNet. Blood 2006;108:1809-20.
19. Thomas J, Wang L, Clark RE, Pirmohamed M. Active transport of imatinib into and out of cells: implications for drug resistance. Blood 2004;104:3739-45.
20. Meissner RDV, Covas DT, Dias PMB, Job F, Leite M, Nardi NB. Analysis of mRNA transcripts in chronic myeloid leukemia patients. Gen Mol Biol 1999;22:475-9.
21. Adler R, Viehmann S, Kuhlisch E, Martiniak Y, Röttgers S, Harbott J, et al. Correlation of BCR/ABL transcript variants with patients' characteristics in childhood chronic myeloid leukaemia. Eur J Haematol 2008;82:112-8.
22. Prejzner W. Relationship of the BCR gene breakpoint and the type of BCR/ABL transcript to clinical course, prognostic indexes and survival in patients with chronic myeloid leukemia. Med Sci Mon 2002;8:193-7.
23. Jaubert J, Martiat P, Dowding C, Ifrah N, Goldman J. The position of the M-BCR breakpoint does not predict the duration of chronic phase or survival in chronic myeloid leukaemia. Br J Haematol 1990;74: 30-5.
24. Tefferi A, Bren G, Wagner K, Schaid D, Ash R, Thibodeau S. The location of the Philadelphia chromosomal breakpoint site and prognosis in chronic granulocytic leukemia. Leukemia 1990;4:839-42.
25. Sokal JE, Cox EB, Baccarani M, Tura S, Gomez GA, Robertson JE, et al. Prognostic discrimination in "good-risk" chronic granulocytic leukemia. Blood 1984;63:789-99.

BCR-ABL1 tyrosine kinase activity at diagnosis, as determined via the pCrkL/CrkL ratio, is predictive of clinical outcome in chronic myeloid leukaemia

Imatinib has become first-line therapy in chronic myeloid leukaemia (CML), but recent studies suggest that more than one-third of patients fail imatinib over 2–3 years. (de Lavallade *et al*, 2008; Lucas *et al*, 2008) It would be clinically helpful to prospectively identify patients unlikely to achieve a complete cytogenetic response (CCRe) following imatinib treatment. Prognostic scoring methods, such as the Sokal score, activity of the imatinib uptake transporter, hOCT1 (human organic cation transporter 1) (Wang *et al*, 2008) and *BCR-ABL1* transcript type (Lucas *et al*, 2009) have been shown to correlate with clinical outcome. However, none of these parameters are sufficiently powerful to reliably prospectively predict patients destined to fare poorly. The phosphorylation status of CrkL has been identified as a surrogate marker of BCR-ABL1 tyrosine kinase activity (White *et al*, 2005), because BCR-ABL1 tyrosine kinase activity cannot be reliability detected in CML patient samples (Patel *et al*, 2007). White *et al* (2005) showed, by Western blotting, that changes in the level of CrkL phosphorylation after 1 month of imatinib treatment correlated with clinical outcome.

In this prospective study we developed a fluorescence-activated cell sorting (FACS) protocol to investigate the predictive value of measuring pCrkL, CrkL and the pCrkL/CrkL ratio (Lucas *et al*, 2009) in fresh peripheral blood from 20 untreated chronic phase CML patients prior to commencing either imatinib or nilotinib treatment. For CrkL phosphorylation determination, cells were resuspended in 500 μl of 2% paraformaldehyde (VWR, Lutterworth, UK) fixed for 10 min at 37°C and chilled on ice for 1 min. Cells were harvested by centrifugation (770 *g*, 3 min), 500 μl 90% methanol (Fisher Scientific, Loughborough, UK) added, vortexed briefly and incubated on ice for 30 min. Cells were then washed (throughout with 1 ml of incubation buffer containing phosphate-buffered saline and 0·5% bovine serum albumin), harvested, and resuspended in 25 μl incubation buffer and incubated at room temperature for 10 min. Antibodies (pCrkL antibody Cat# 3181 lot 3 and 4, Cell Signalling Technology, Danvers, MA, USA; CrkL Cat#sc-319, Santa Cruz Biotechnology, Santa Cruz, CA, USA; control anti-normal-rabbit immunoglobulins Cat#AB-105-C, R&D Systems, Abingdon, UK) were added to a final concentration of 28 μg/ml, vortexed and incubated at room temperature for 40 min then washed twice, resuspended in fluorescein-labelled goat-anti-rabbit second layer antibody Alexa Fluor 488 (10 μg/ml; Invitrogen, Paisley, UK), and incubated at room temperature in the dark for 30 min. Twice washed cells were analysed using flow cytometry (FACScalibur; Becton Dickinson, Oxford, UK), with CELLQUEST PRO software (Becton Dickinson) for data analysis. The levels of pCrkL and CrkL present in the sample were determined as the geometric mean fluorescence intensity (MFI) minus the MFI value of the control sample. The pCrKL/CrKL ratio of a sample was determined via:

$$\text{pCrkL/CrkL ratio} = \frac{\text{pCrkL} - \text{Control MFI}}{\text{CrkL} - \text{Control MFI}} \times 100$$

Flow cytometry has the advantage that it requires fewer patient cells ($\sim 10^3$ cells) than Western blotting, produces quantitative values, and results can be obtained rapidly. Additionally this methodology could be utilized more easily at other laboratories. A representative FACS histogram of a patient sample is shown in Fig 1.

Our study demonstrated that measuring pCrkL levels alone did not correlate with clinical outcome (Fig 2A). Hundred per cent of patients with a pre-treatment pCrkL/CrkL ratio <25 achieved CCRe following 12 months of imatinib treatment (Fig 2B). Of these, 75% of patients achieved their CCRe within 6 months. No patient whose pCrkL/CrkL ratio was greater than 25 achieved CCRe following 12 months of imatinib treatment ($P = 0{\cdot}0003$).

Six out of 14 imatinib-treated patients failed to achieve a CCRe following imatinib treatment. Three switched to the second generation tyrosine kinase inhibitor nilotinib and the other three remain on imatinib. No BCR-ABL1 kinase domain mutations were detected in patients who failed to achieve a CCRe by 12 months. The remaining eight patients, with a diagnostic pCrkL/CrkL ratio below 25 and a maximum follow up of 2 years, remain in CCRe.

During this study six patients commenced nilotinib treatment at initial diagnosis. Their pCrkL/CrkL MFI ratios at diagnosis ranged from 7·5 to 47·3 Fig 2C. Following 12 months of treatment all achieved a complete cytogenetic response. Patients with a pCrkL/CrkL MFI ratio greater than 25 who were treated with nilotinib achieved a CCRe compared to 0% of imatinib-treated patients ($P = 0{\cdot}03$). This is probably because nilotinib is more potent BCR-ABL1 tyrosine kinase inhibitor (O'Hare *et al*, 2005). These data suggest that patients with high BCR-ABL1 tyrosine kinase activity (pCrkL/CrkL

doi:10.1111/j.1365-2141.2009.08066.x

(A)

		Total	Imatinib-treated	Nilotinib-treated
Number of patients		20	14	6
Age years (range)		48 (19–72)	48 (19–72)	56 (44–67)
Sex (M/F)		9/11	7/7	2/4
Sokal score	High	8	5	3
	Intermediate	6	3	3
	Low	6	6	0

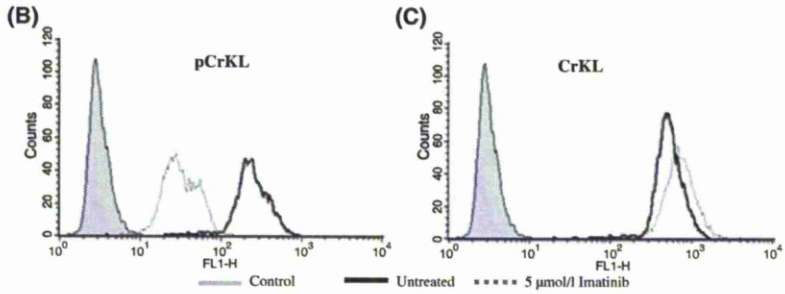
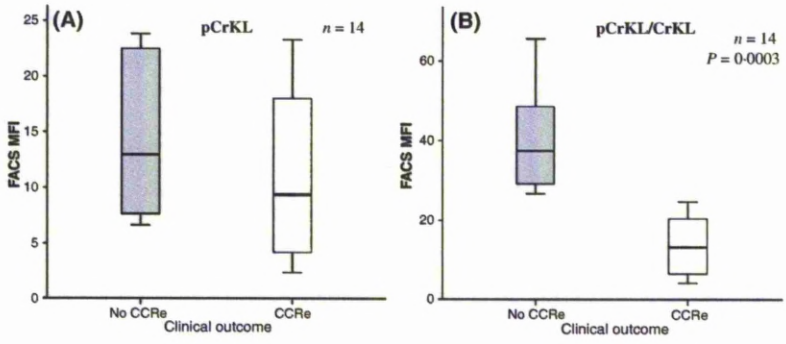


Fig 1. Patient characteristics and optimization of pCrKL and CrKL detection by flow cytometry. (A) Summary of patients characteristics. (B) Representative FACS plot showing pCrKL and CrKL (C) in a newly diagnosed CML patient and changes following *in vitro* treatment with 5 μmol/l imatinib for 24 h.



(C)

TKI treatment	pCrKL/CrKL ratio	CCRe at 12 months
Imatinib	4·13	Yes
Imatinib	6·05	Yes
Imatinib	7·01	Yes
Imatinib	11·67	Yes
Imatinib	14·77	Yes
Imatinib	16·19	Yes
Imatinib	24·5	Yes
Imatinib	24·74	Yes
Imatinib	26·68	No
Imatinib	29·13	No
Imatinib	35·29	No
Imatinib	39·57	No
Imatinib	48·62	No
Imatinib	65·70	No
Nilotinib	7·49	Yes
Nilotinib	12·31	Yes
Nilotinib	13·78	Yes
Nilotinib	18·71	Yes
Nilotinib	34·72	Yes
Nilotinib	47·3	Yes

Fig 2. pCrKL, CrKL and pCrKL/CrKL ratio level at diagnosis and clinical outcome. FACS assessment of pCrKL (A) and pCrKL/CrKL ratio (B) in untreated newly diagnosed CML patients, stratified by clinical outcome following 12 months of imatinib treatment. (C) pCrKL/CrKL ratio at diagnosis and clinical outcome at 12 months for imatinib- and nilotinib-treated patients. Patients with a pCrKL/CrKL ratio below 25 responded to imatinib treatment.

MFI ratio >25) have a low probability of responding to imatinib, but may nevertheless achieve a complete cytogenetic response on nilotinib treatment. All nilotinib-treated patients remained in CCRe with continued treatment.

In addition, serial monitoring of the trends in the pCrkL/CrkL ratio following commencement of imatinib treatment had no predictive value. We analysed the diagnostic pCrkL/CrkL ratio data for both imatinib- and nilotinib-treated

patients in relation to other clinical parameters such as white blood cell count at diagnosis, Sokal score and hOCT1 (for imatinib-treated patients only), (Davies *et al*, 2009) and found no correlation. This suggests that the pCrkL/CrkL ratio is an independent biomarker of clinical outcome for imatinib treated patients.

It is interesting to speculate that the response to imatinib therapy is related to BCR-ABL1 tyrosine kinase activity present at diagnosis. A fixed dose of imatinib may therefore only be able to suppress the lower kinase activity i.e. pCrkL/CrkL ratio <25; while nilotinib, which is known to be a more potent BCR-ABL1 kinase inhibitor than imatinib (Kantarjian *et al*, 2007), may suppress the BCR-ABL1 tyrosine kinase activity to a greater proportional extent. This may explain the higher incidence of CCRe following treatment with nilotinib.

In conclusion, the pCrkL/CrkL ratio prior to commencement of treatment is a potent predictor of clinical outcome in patients treated with imatinib. Its role as a predictive biomarker of response to tyrosine kinase inhibitor therapy merits further investigation in larger series, though it is essential that this is performed on freshly isolated cells prior to any treatment.

Acknowledgements

The authors would like to thank the University of Liverpool Biobank staff Joanna Middleton, Melanie Oates and Alison Hayes for their help and support during this project.

Authorship and disclosures

CML, RJH and REC designed the study and wrote the manuscript. CML and AG performed the laboratory work for this study. KK and SW were responsible for taking patient samples and providing clinical information. REC was the principal investigator. The authors reported no potential conflicts of interest.

Claire M. Lucas
Robert J. Harris
Athina Giannoudis
Katy Knight
Sarah J. Watmough
Richard E. Clark

Department of Haematology, Royal Liverpool University Hospital, Prescot Street, Liverpool L7 8XP, UK.
E-mail: r.e.clark@liv.ac.uk

References

Davies, A., Jordanides, N.E., Giannoudis, A., Lucas, C.M., Hatziieremia, S., Harris, R.J., Jørgensen, H.G., Holyoake, T.L., Pirmohamed, M., Clark, R.E. & C., M.J. (2009) Nilotinib concentration in cell lines and primary CD34+ chronic myeloid leukemia cells is not mediated by active uptake or efflux by major drug transporters. *Leukemia*, **23**, 1999–2006.

Kantarjian, H.M., Giles, F., Gattermann, N., Bhalla, K., Alimena, G., Palandri, F., Ossenkoppele, G.J., Nicolini, F.-E., O'Brien, S.G., Litzow, M., Bhatia, R., Cervantes, F., Haque, A., Shou, Y., Resta, D.J., Weitzman, A., Hochhaus, A. & le Coutre, P. (2007) Nilotinib (formerly AMN107), a highly selective Bcr-Abl tyrosine kinase inhibitor, is effective in patients with Philadelphia chromosome-positive chronic myelogenous leukemia in chronic phase following imatinib resistance and intolerance. *Blood*, **110**, 3540–3546.

de Lavallade, H., Apperley, J.F., Khorashad, J.S., Milojkovic, D., Reid, A.G., Bua, M., Szydlo, R., Olavarria, E., Kaeda, J., Goldman, J.M. & Marin, D. (2008) Imatinib for newly diagnosed patients with chronic myeloid leukemia: incidence of sustained responses in an intention-to-treat analysis. *Journal of Clinical Oncology*, **26**, 3358–3363.

Lucas, C.M., Wang, L., Austin, G.M., Knight, K., Watmough, S.J., Shwe, K.H., Dasgupta, R., Butt, N.M., Galvani, D., Hoyle, C.F., Seale, J.R.C. & Clark, R.E. (2008) A population study of imatinib in chronic myeloid leukaemia demonstrates lower efficacy than in clinical trials. *Leukemia*, **22**, 1963–1966.

Lucas, C.M., Harris, R.J., Giannoudis, A., Davies, A., Knight, K., Watmough, S.J., Wang., L. & Clark, R.E. (2009) Chronic myeloid leukaemia patients with the e13a2 BCR-ABL fusion transcript have inferior responses to imatinib than e14a2 patients. *Haematologica*, **94**, 1362–1367.

O'Hare, T., Walters, D.K., Stoffregen, E.P., Jia, T., Manley, P.W., Mestan, J., Cowan-Jacob, S.W., Lee, F.Y., Heinrich, M.C., Deininger, M.W.N. & Druker, B.J. (2005) *In vitro* Activity of Bcr-Abl Inhibitors AMN107 and BMS-354825 against Clinically Relevant Imatinib-Resistant Abl Kinase Domain Mutants. *Cancer Research*, **65**, 4500–4505.

Patel, H., Marley, S.B. & Gordon, M.Y. (2007) Conventional Western blotting techniques will not reliably quantify p210BCR-ABL1 levels in CML mononuclear cells. *Blood*, **109**, 1335.

Wang, L., Giannoudis, A., Lane, S., Williamson, P., Pirmohamed, M. & Clark, R.E. (2008) Expression of the Uptake Drug Transporter hOCT1 is an Important Clinical Determinant of the Response to Imatinib in Chronic Myeloid Leukemia. *Clinical Pharmacology and Therapeutics*, **83**, 258–264.

White, D., Saunders, V., Lyons, A.B., Branford, S., Grigg, A., To, L.B. & Hughes, T. (2005) *In vitro* sensitivity to imatinib-induced inhibition of ABL kinase activity is predictive of molecular response in patients with de novo CML. *Blood*, **106**, 2520–2526.

Keywords: imatinib, pCrkL, pCrkL/CrKL ratio, CML, biomarker.

Leukemia (2008), 1–4

www.nature.com/leu

LETTER TO THE EDITOR

A population study of imatinib in chronic myeloid leukaemia demonstrates lower efficacy than in clinical trials

Leukemia advance online publication, 28 August 2008; doi:10.1038/leu.2008.225

Imatinib is a tyrosine kinase inhibitor that has undoubtedly saved and improved the quality of lives of many thousands of patients with chronic myeloid leukaemia (CML). It has become the treatment of choice for newly diagnosed patients in chronic phase, and a recent report suggests that modern drug treatment may produce superior results to allogeneic stem cell transplantation (SCT).[1] The imatinib-treated cohort within the International Randomized Study of Interferon and STI571 (IRIS) has provided valuable information on the longer term effects of imatinib treatment. On 400 mg daily, the overall survival at 5 years is 89%.[2] Furthermore, the IRIS data suggest that the rate of disease progression may decrease with time. However, these data are derived from a clinical trial, and it is therefore important to establish if these results can be reproduced in a more general CML population. Here we present the results of a population-based study of CML treated with 400 mg daily imatinib in our health-care area.

In our area of the North West of England, the adjacent North Wales coastal strip and the Isle of Man (total population 2 million), all services for adults with haematological cancer are located at 12 hospitals. Molecular diagnosis of CML and monitoring for *BCR-ABL* transcripts are carried out at a single centre (RLUH) for all CML patients in this geographical area. We are therefore able to trace the clinical course of every CML patient in our area.

All patients aged 16 years or more with CML newly diagnosed between 1 January 2003 and 30 June 2006 are included in this study. Younger patients were excluded as imatinib usage in children is not universal, and earlier patients were excluded as the timing of imatinib approval in 2002 varied across our geographical area. Patients were included in the assessment of imatinib efficacy if they received imatinib 400 mg daily from original diagnosis (preceded only by up to 6 weeks of hydroxycarbamide).

Considerable effort was made to include all CML cases, first from the records at the molecular diagnostic centre and second through the specific enquiry of local haematologists. Local haematologists verified that each case had never received prior interferon-α or alternative chemotherapy.

Responses were defined conventionally:[3] complete haematological response (CHR) = normalization of the blood count and resolution of splenomegaly; complete cytogenetic response (CCR) = no Ph^+ metaphases among at least 20 marrow metaphases or a *BCR-ABL/ABL* ratio of 1% or less,[4] which we have previously shown to be tightly correlated with cytogenetically defined CCR; major molecular response (MMR) = a *BCR-ABL/ABL* ratio of 0.1% or less. Measurement of *BCR-ABL* transcripts was carried out as described earlier using quantitative real-time PCR.[4,5] In some cases, serial cytogenetic data were not available and achievement of CCR is based on a BCR-ABL/ABL transcript ratio of below 1%; we have therefore used the term 'CCR equivalence' (CCRe) to encompass these cases.

Over the 42 months of the study, 88 new cases of CML were seen, giving an annual incidence of 1.2 cases per 100 000 population per annum. These comprised 41 men and 47 women, and their median age at diagnosis was 53.4 years (range 18.6–86.8 years). Of these, four presented in blast crisis and were treated with chemotherapy before imatinib and then SCT. Of the 84 presenting in chronic phase, 16 cases were not assessable for the effect of imatinib as they first received interferon-α (seven cases) or an elective SCT (one case); or because insufficient follow-up data were available (eight cases).

A flow diagram of patient outcome is given in Figure 1. During the first 12 months of treatment, 3 of the 68 patients who received imatinib from original diagnosis progressed to blast crisis, of whom two died and one achieved a second chronic phase during which she underwent SCT. Two patients had serious side effects attributable to imatinib (one Stevens-Johnson syndrome, one hepatotoxicity) and were successfully treated with an alternative tyrosine kinase inhibitor. One further patient lost an initial CHR and proceeded to SCT. Six cases therefore failed imatinib before 12 months. Of the 62 cases that were assessable for response at 12 months, 28 had achieved CCRe.

Twenty-three of these 28 cytogenetic responders (CCRe) were assessable at 18 months of imatinib treatment, and 22 remained in CCRe; the remaining case developed central nervous system blast crisis. Of the 34 cases who had not achieved CCRe at 12 months, eight patients achieved CCRe at 18 months.

Twelve of the original 68 imatinib-treated patients were unassessable at 18 months (three cases lost their CHR and proceeded to SCT, two died of disease progression and seven cases had not reached the 18-month assessment point). By 24 months, a total of six of the original 68 imatinib-treated patients had progressed to advanced phase (9%), and 10 further cases remained in chronic phase but had received SCT or switched to an alternative tyrosine kinase inhibitor, because of intolerance (three cases) or failure to achieve/maintain CCRe. The flow diagram for all imatinib-treated patients is given in Figure 1.

The overall CCRe rates were 41% (28 of 68 patients) at 12 months, 49% (30 of 61 assessable cases) at 18 months and 51% (28 of 55 assessable cases) at 24 months (Table 1). MMR rates were 22% at 12 months, 26% at 18 months and 31% at 24 months.

Using recommended criteria,[3] at 24 months, 49% (27 of 55 assessable cases) had failed imatinib because of either progression to blast crisis (six cases; all fatal), failure to achieve/maintain CCRe (19 cases, of which four lost CHR as well) or intolerance (two cases). Fourteen of the 15 failures who remain in CHR and without disease progression have achieved and remain in CCRe at latest follow-up, following either SCT or an alternative tyrosine kinase inhibitor. Figure 2 gives the event-free survival rate according to Sokal score; no statistically significant difference is seen.

Despite several hundred publications on various clinical aspects of imatinib, there are, to our knowledge, only three

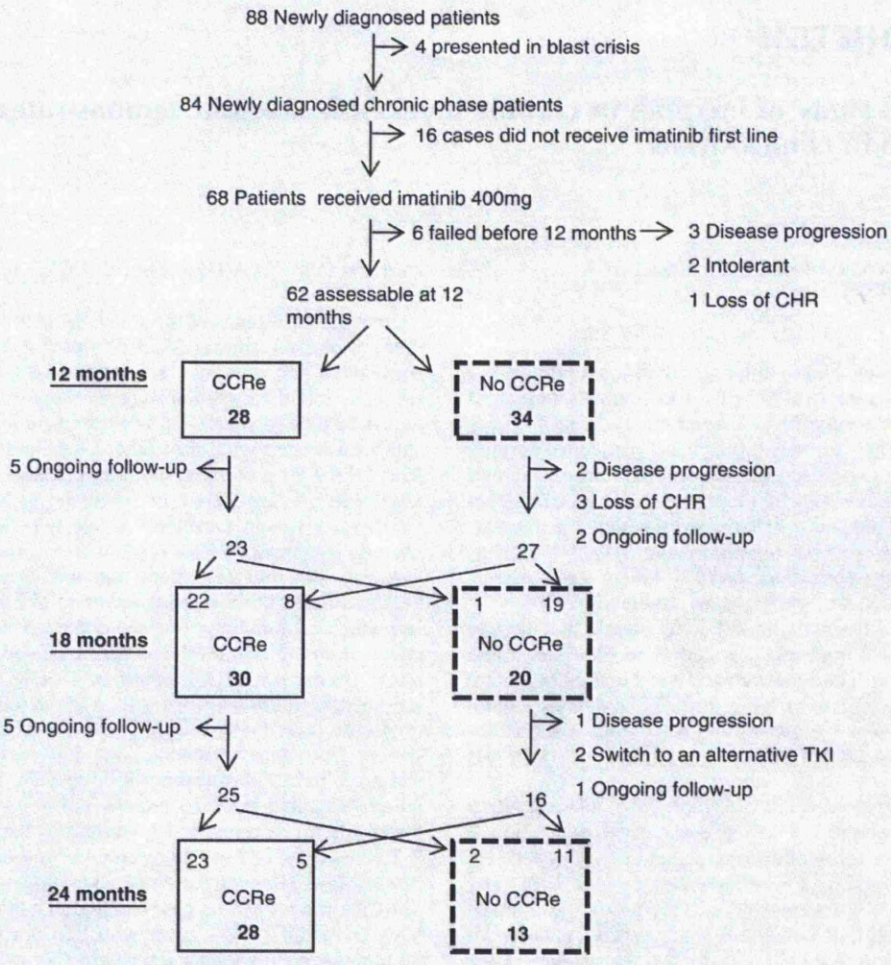


Figure 1 A flow diagram of patient outcome. Abbreviations are as defined in the text.

Table 1 CCRe response rate for all 88 patients and the 68 patients who received imatinib from initial diagnosis

	All patients				*Patients receiving imatinib from initial diagnosis*			
	Total	*12 months*	*18 months*	*24 months*	*Total*	*12 months*	*18 months*	*24 months*
Number of cases assessable	88	83	81	80		68	61	55
Low	20	55% (11/20)	53% (10/53)	42% (8/19)	18	50% (9/18)	52% (9/17)	43% (7/16)
Intermediate	29	24% (6/25)	36% (9/25)	36% (9/25)	22	27% (6/22)	38% (6/21)	42% (8/19)
High	28	48% (13/27)	41% (11/27)	41% (11/27)	23	52% (12/23)	61% (11/18)	68% (11/16)
No data	11	27% (3/11)	40% (4/10)	44% (4/9)	5	20% (1/5)	40% (2/5)	50% (2/4)
Overall CCRe	88	40% (33/83)	42% (34/81)	40% (32/80)	68	41% (28/68)	49% (30/61)	51% (28/55)

Abbreviation: CCRe, complete cytogenetic response equivalence.
The response rate is also stratified according to Sokal score.

series describing results in previously untreated patients. The IRIS trial demonstrated that after 5 years of imatinib therapy, the overall survival of patients was 89%, with 87% achieving a CCR.[2] In a separate study of 279 newly diagnosed patients presenting to the MD Anderson Hospital, 87% achieved a CCR, with an estimated 3-year survival rate of 96%.[6] In a recent report in abstract form on 207 consecutive patients referred to the Hammersmith Hospital in London, the 4-year progression-free survival was estimated at 90%, with 34 (16%) discontinuing imatinib while still in chronic phase. The cumulative incidence of CCR and MMR was 59 and 12% by 12 months, and 87 and 58% by 48 months, respectively. The cumulative incidence of loss of CCR at 48 months was 17%.[7] None of these three studies were population-based.

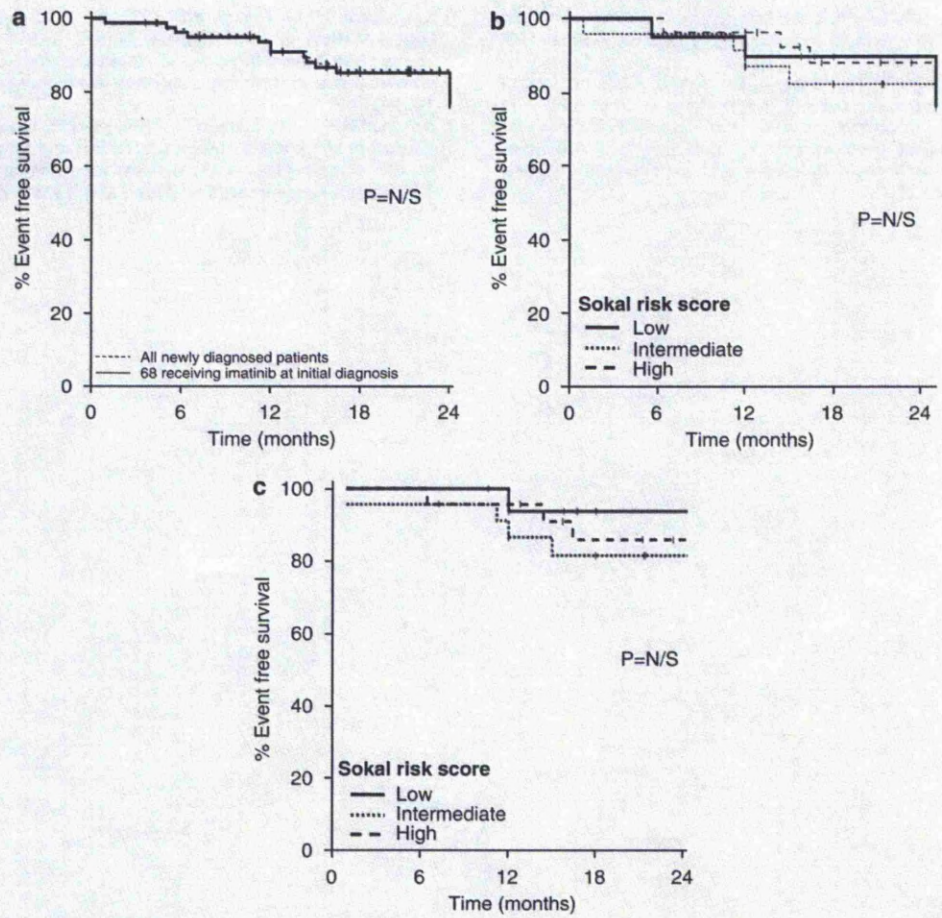


Figure 2 Kaplan–Meier estimates of event-free survival. (**a**) Comparison between all 88 patients and the 68 patients who received imatinib from initial diagnosis. Remaining panels: outcome according to Sokal score for all 88 patients (**b**) and for the 68 patients (**c**). No statistically significant differences were observed (SPSS statistical package–antel-Cox log-rank test).

In contrast, our population-based study shows that, by using the recommended criteria,[3] at 24 months 49% (27 of 55 assessable cases) had failed or were intolerant of imatinib. The data suggest that caution is needed in extrapolating clinical trial data to the general CML population.

Acknowledgements

We thank Dr RJ Harris for all his help and support during this project. REC has received research support and has participated in advisory boards for Novartis. No author has any competing financial interests.

CM Lucas[1], L Wang[1], GM Austin[1], K Knight[1], SJ Watmough[1], KH Shwe[2], R Dasgupta[3], Nauman M Butt[4], D Galvani[4], CF Hoyle[5], JRC Seale[6] and RE Clark[1]
[1]Department of Haematology, Royal Liverpool University Hospital, Prescot St, Liverpool, UK;
[2]Department of Haematology, Warrington Hospital, Warrington, Cheshire, UK;
[3]Department of Haematology, Aintree University Hospital, Liverpool, UK;
[4]Department of Haematology, Arrowe Park Hospital, Upton, Wirral, Merseyside, UK;
[5]Department of Haematology, Ysbyty Glan Clywd, Rhyl, Denbighshire, UK and
[6]Department of Haematology, Ysbyty Gwynedd, Bangor, Gwynedd, UK
E-mail: clarkre@liverpool.ac.uk

References

1 Hehlmann R, Berger U, Pfirrmann M, Heimpel H, Hochhaus A, Hasford J *et al.* Drug treatment is superior to allografting as first-line therapy in chronic myeloid leukemia. *Blood* 2007; **109**: 4686–4692.
2 Druker BJ, Guilhot F, O'Brien SG, Gathmann I, Kantarjian H, Gattermann N *et al.* Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. *N Engl J Med* 2006; **355**: 2408–2417.
3 Baccarani M, Saglio G, Goldman J, Hochhaus A, Simonsson B, Appelbaum F *et al.* Evolving concepts in the management of chronic myeloid leukemia: recommendations from an expert panel on behalf of the European LeukemiaNet. *Blood* 2006; **108**: 1809–1820.
4 Wang L, Pearson K, Pillitteri L, Ferguson JE, Clark RE. Serial monitoring of BCR-ABL by peripheral blood real-time polymerase

chain reaction predicts the marrow cytogenetic response to imatinib mesylate in chronic myeloid leukaemia. *Br J Haematol* 2002; **118**: 771–777.

5 Hughes T, Deininger M, Hochhaus A, Branford S, Radich J, Kaeda J *et al.* Monitoring CML patients responding to treatment with tyrosine kinase inhibitors: review and recommendations for harmonizing current methodology for detecting BCR-ABL transcripts and kinase domain mutations and for expressing results. *Blood* 2006; **108**: 28–37.

6 Kantarjian HM, Talpaz M, O'Brien S, Jones D, Giles F, Garcia-Manero G *et al.* Survival benefit with imatinib mesylate versus interferon-{alpha}-based regimens in newly diagnosed chronic-phase chronic myelogenous leukemia. *Blood* 2006; **108**: 1835–1840.

7 de Lavallade H, Milojkovic D, Khorashad JS, Reid A, Olavarria E, Kaeda J *et al.* Outcome, prognostic factors and long-term follow-up in 207 chronic phase CML patients receiving front-line imatinib 400 mg at a single institution. *Blood* 2007; **110**, abstract 1045.

REFERENCES

1. Bennett JH. Case of hypertrophy of the spleen and liver in which death took place from suppuration of the blood. *Edinburgh Medical Surgery* 1845;64:413-423.
2. Virchow R. Weisses blut. *Frorieps Notizen* 1845 36:151-156.
3. Geary CG. The story of chronic myeloid leukaemia. *British Journal of Haematology.* 2000;110(1):2-11.
4. Goldman JM, Daley GQ. Chronic Myeloid Leukemia - A Brief History. Myeloproliferative Disorders: Springer Berlin Heidelberg; 2007:1-13.
5. Neumann E. Ein Fall Von Leukämie Mit Erkrankung Des Knochenmarks *Archive der Heilkunde.* 1870
6. Ehrlich P. Farbenanalytische Untersuchungen zur Histologie und Klinik des Blutes. *Hirschwald, Berlin.* 1891
7. Nowell P, Hungerford D. A minute chromosome in human chronic granulocytic leukemia. *Science.* 1960 132:1497.
8. Rowley JD. A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. *Nature.* 1973;243:290-293.
9. Abelson HT, Rabstein LS. Lymphosarcoma: Virus-induced Thymic-independent Disease in Mice. *Cancer Research.* 1970;30(8):2213-2222.
10. Bartram CR, de Klein A, Hagemeijer A, van Agthoven T, Geurts van Kessel A, Bootsma D. Translocation of c-ab1 oncogene correlates with the presence of a Philadelphia chromosome in chronic myelocytic leukaemia. *Nature.* 1983;306:277-280.
11. Groffen J, Stephenson JR, Heisterkamp N, de Klein A, Bartram CR, Grosveld G. Philadelphia chromosomal breakpoints are clustered within a limited region, bcr, on chromosome 22. *Cell.* 1984;36:93-99.
12. Lugo TG, Pendergast AM, Muller AJ, Witte ON. Tyrosine kinase activity and transformation potency of bcr-abl oncogene products. *Science* 1990;247(4946):1079-1082.
13. Daley G, Van Etten R, Baltimore D. Induction of chronic myelogenous leukemia in mice by the P210bcr/abl gene of the Philadelphia chromosome. *Science.* 1990;247:824-830.
14. Heisterkamp N. Acute leukemia in bcr/abl transgenic mice. *Nature.* 1990;344:251-253.
15. Druker BJ, Tamura S, Buchdunger E, et al. Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. *Nature Medicine.* 1996;2(5):561-566.
16. Druker BJ. Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. *Nature Med.* 1996;2:561-566.
17. O'Brien SG, Guilhot F, Larson RA, et al. Imatinib Compared with Interferon and Low-Dose Cytarabine for Newly Diagnosed Chronic-Phase Chronic Myeloid Leukemia. *New England Journal of Medicine.* 2003;348(11):994-1004.
18. Druker BJ, Guilhot F, O'Brien SG, et al. Five-Year Follow-up of Patients Receiving Imatinib for Chronic Myeloid Leukemia. *New England Journal of Medicine.* 2006;355(23):2408-2417.
19. Sessions J. Chronic myeloid leukemia in 2007. *America Journal of Health-System Pharmacy.* 2007;64(24, Supplement 15):S4-9.
20. Sawyers CL. Chronic Myeloid Leukemia. *New England Journal of Medicine.* 1999;340(17):1330-1340.
21. Lucas CM, Wang L, Austin GM, et al. A population study of imatinib in chronic myeloid leukaemia demonstrates lower efficacy than in clinical trials. *Leukemia.* 2008;22(10):1963-1966.

22. Cortes J. Natural history and staging of chronic myelogenous leukemia. *Hematology/Oncology Clinics of North America*. 2004;18(3):569-584.
23. Deininger MWN, Druker BJ. Specific Targeted Therapy of Chronic Myelogenous Leukemia with Imatinib. *Pharmacological Reviews*. 2003;55(3):401-423.
24. Heyssel R, Brill BA, Woodbury LA, et al. Leukemia in Hiroshima Atomic Bomb Survivors. *Blood*. 1960;15(3):313-331.
25. Corso A, Lazzarino M, E M, et al. Chronic myelogenous leukemia and exposure to ionizing radiation-a retrospective study of 443 patients. *Annals of Hematology* 1995;70:79-82.
26. Deininger MWN, Bose S, Gora-Tybor J, Yan X-H, Goldman JM, Melo JV. Selective Induction of Leukemia-associated Fusion Genes by High-Dose Ionizing Radiation. *Cancer Research*. 1998;58(3):421-425.
27. Moloney WC. Radiogenic leukemia revisited. *Blood*. 1987;70(4):905-908.
28. Cervantes F, Hernandez-Boluda J, Ferrer A, Cid J, Montserrat E. The changing profile of Ph-positive chronic myeloid leukemia at presentation: possible impact of earlier diagnosis on survival. *Haematologica*. 1999;84(4):324-327.
29. Ganesan T, Rassool F, Guo A, et al. Rearrangement of the bcr gene in Philadelphia chromosome-negative chronic myeloid leukemia. *Blood*. 1986;68(4):957-960.
30. Schoch C, Schnittger S, Bursch S, et al. Comparison of chromosome banding analysis, interphase- and hypermetaphase-FISH, qualitative and quantitative PCR for diagnosis and for follow-up in chronic myeloid leukemia: a study on 350 cases. *Leukemia*. 2002;16(1):53-59.
31. Kohno S-I, Abe S, Sandberg AA. The chromosomes and causation of human cancer and leukemia: XXXVIII. Cytogenetic experience in Ph1-negative chronic myelocytic leukemia (CML). *American Journal of Hematology*. 1979;7(3):281-291.
32. Woodring PJ, Hunter T, Wang JY. Regulation of F-actin-dependent processes by the Abl family of tyrosine kinases. *Journal of Cell Scienec*. 2003;116:2613-2626.
33. Baccarani M, Saglio G, Goldman J, et al. Evolving concepts in the management of chronic myeloid leukemia: recommendations from an expert panel on behalf of the European LeukemiaNet. *Blood*. 2006;108(6):1809-1820.
34. Feller SM, Knudsen B, H. H. c-Abl kinase regulates the protein binding activity of c-Crk. *The EMBO Journal* 1994;13(10):2341-2351.
35. Ren R. Mechanisms of BCR-ABL in the pathogenesis of chronic myelogenous leukaemia. *Nature Reviews Cancer*. 2005;5(3):172-183.
36. Goldman JM, Melo JV. Chronic Myeloid Leukemia -- Advances in Biology and New Approaches to Treatment. *The New England Journal of Medicine*. 2003;349(15):1451-1464.
37. Deininger MW, Goldman JM, Melo JV. The molecular biology of chronic myeloid leukemia. *Blood*. 2000;96:3343-3356.
38. Collins S, Coleman H, Groudine M. Expression of bcr and bcr-abl fusion transcripts in normal and leukemic cells. *Molecular and Cell Biology*. 1987;7(8):2870-2876.
39. Reuther GW, Fu H, Cripe LD, Collier RJ, Pendergast AM. Association of the protein kinases c-Bcr and Bcr-Abl with proteins of the 14-3-3 family. *Science*. 1994;266(5182):129-133.
40. Ron D, Zannini M, Lewis M, et al. A region of proto-dbl essential for its transforming activity shows sequence similarity to a yeast cell cycle gene, CDC24, and the human breakpoint cluster gene, bcr. *The new Biologist*. 1991;3(4):372-379.
41. Montaner S, Perona R, Saniger L, Lacal JC. Multiple Signalling Pathways Lead to the Activation of the Nuclear Factor κB by the Rho Family of GTPases. *Journal of Biological Chemistry*. 1998;273(21):12779-12785.

42. Voncken JW. Increased neutrophil respiratory burst in bcr-null mutants. *Cell.* 1995;80:719-728.
43. Sattler M, Mohi MG, Pride YB, et al. Critical role for Gab2 in transformation by BCR/ABL. *Cancer Cell.* 2002;1(5):479-492.
44. Million RP, Van Etten RA. The Grb2 binding site is required for the induction of chronic myeloid leukemia-like disease in mice by the Bcr/Abl tyrosine kinase. *Blood.* 2000;96:664-670.
45. Pendergast AM, Quilliam LA, Cripe LD, et al. BCR-ABL-induced oncogenesis is mediated by direct interaction with the SH2 domain of the GRB-2 adaptor protein. *Cell.* 1993;75(1):175-185.
46. Goga A, McLaughlin J, Afar DE, Saffran DC, Witte ON. Alternative signals to RAS for hematopoietic transformation by the Bcr-Abl oncogene. *Cell.* 1995;82:981-988.
47. Ben-Neriah Y, Daley G Q, Mes-Masson A M, Witte O N, Baltimore D. The chronic myelogenous leukemia-specific p210 protein is the product of the BCR/ABL hybrid gene. *Science.* 1986;233:212–214.
48. Pasternak G, Hochhaus A, Schultheis B, Hehlmann R. Chronic myelogenous leukemia: molecular and cellular. *Journal of Cancer Research and Clinical Oncology.* 1998;124(12):643-660.
49. Ravandi F, Cortes J, Albitar M, et al. Chronic myelogenous leukaemia with p185(BCR/ABL) expression: characteristics and clinical significance. *British Journal of Haematology.* 1999;107(3):581-586.
50. Quintas-Cardama A, Kantarjian H, Cortes J. Flying under the radar: the new wave of BCR-ABL inhibitors. *Nat Rev Drug Discov.* 2007;6(10):834-848.
51. Melo J, Myint H, Galton D, Goldman J. P190BCR-ABL chronic myeloid leukaemia: the missing link with chronic myelomonocytic leukaemia? *Leukemia.* 1994;8(1):208-211.
52. Melo JV. BCR-ABL gene variants. *Bailliere's Clinical Haematology.* 1997;10(2):203-222.
53. Hochhaus A, Reiter A, Skladny H, et al. A novel BCR-ABL fusion gene (e6a2) in a patient with Philadelphia chromosome-negative chronic myelogenous leukemia. *Blood.* 1996;88(6):2236-2240.
54. Morris SW, Daniel L, Ahmed CM, Elias A, Lebowitz P. Relationship of bcr breakpoint to chronic phase duration, survival, and blast crisis lineage in chronic myelogenous leukemia patients presenting in early chronic phase *Blood.* 1990;75(10):2035-2041.
55. Polampalli S, Choughule N, Negi N, et al. Analysis and comparison of clinicohematological parameters and molecular and cytogenetic response of two Bcr/Abl fusion transcripts. *Genetics and Molecular Research.* 2008;7(4):1138-1149.
56. Rosas-Cabral A, Martínez-Mancilla M, Ayala-Sánchez M, et al. Analysis of Bcr-abl type transcript and its relationship with platelet count in Mexican patients with chronic myeloid leukemia. *Gaceta médica de México.* 2003;139(6):553-559.
57. Meissner RDV, Dias PM, Covas DT, Job F, Leite M, Nardi NB. A polymorphism in exon b2 of the major breakpoint cluster region (M-bcr) identified in chronic myeloid leukaemia patients. *British Journal of Haematology.* 1998;103(1):224-226.
58. Perego RA, Costantini M, Cornacchini G, et al. The possible influences of B2A2 and B3A2 BCR/ABL protein structure on thrombopoiesis in chronic myeloid leukaemia. *European Journal of Cancer.* 2000;36(11):1395-1401.
59. Shepherd P, Suffolk R, Halsey J, Allan N. Analysis of molecular breakpoint and m-RNA transcripts in a prospective randomized trial of interferon in chronic myeloid leukaemia: no correlation with clinical features, cytogenetic response, duration of chronic phase, or survival. *British Journal of Haematology.* 1995;89(3):546-554.

60. Mills KI, Benn P, Birnie GD. Does the breakpoint within the major breakpoint cluster region (M-bcr) influence the duration of the chronic phase in chronic myeloid leukemia? An analytical comparison of current literature. *Blood.* 1991;78(5):1155-1161.
61. de Lemos J, de Oliveira C, Scerni A, et al. Differential molecular response of the transcripts B2A2 and B3A2 to imatinib mesylate in chronic myeloid leukemia. *Genetics and Molecular Research.* 2005;30(4):803-811.
62. Vega-Ruiz A, Kantarjian H, Shan J, et al. Better Molecular Response to Imatinib for Patients (pts) with Chronic Myeloid Leukemia (CML) in Chronic Phase (CP) Carrying the b3a2 Transcript Compared to b2a2. *ASH Annual Meeting Abstracts.* 2007;110(11):1939.
63. Daley GQ, Baltimore D. Transformation of an Interleukin 3-Dependent Hematopoietic Cell Line by the Chronic Myelogenous Leukemia-Specific P210bcr/abl Protein. *Proceedings of the National Academy of Sciences.* 1988;85(23):9312-9316.
64. Barnes DJ, Schultheis B, Adedeji S, Melo JV. Dose-dependent effects of Bcr-Abl in cell line models of different stages of chronic myeloid leukemia. *Oncogene.* 2005;24(42):6432-6440.
65. Zhang X, Ren R. Bcr-Abl efficiently induces a myeloproliferative disease and production of excess interleukin-3 and granulocyte-macrophage colony-stimulating factor in mice: a novel model for chronic myelogenous leukemia. *Blood.* 1998;92:3829-3840.
66. Jonuleit T, Peschel C, Schwab R, et al. Bcr-Abl kinase promotes cell cycle entry of primary myeloid CML cells in the absence of growth factors. *British Journal of Haematology.* 1998;100(2):295-303.
67. Benekli M, Baer MR, Baumann H, Wetzler M. Signal transducer and activator of transcription proteins in leukemias. *Blood.* 2003;101(8):2940-2954.
68. de Groot RP, Raaijmakers JAM, Lammers J-WJ, Koenderman L. STAT5-Dependent CyclinD1 and Bcl-xL Expression in Bcr-Abl-Transformed Cells. *Molecular Cell Biology Research Communications.* 2000;3(5):299-305.
69. Jilani I, Kantarjian H, Gorre M, et al. Phosphorylation levels of BCR-ABL, CrkL, AKT and STAT5 in imatinib-resistant chronic myeloid leukemia cells implicate alternative pathway usage as a survival strategy. *Leukemia Research.* 2008;32(4):643-649.
70. Samanta A, Perazzona B, Chakraborty S, et al. Janus kinase 2 regulates Bcr-Abl signaling in chronic myeloid leukemia. *Leukemia.* 2011;25(3):463-472.
71. Bedi A, Zehnbauer BA, Barber JP, Sharkis SJ, Jones RJ. Inhibition of apoptosis by BCR-ABL in chronic myeloid leukemia. *Blood.* 1994;83(8):2038-2044.
72. Bedi A, Barber JP, Bedi GC, et al. BCR-ABL-mediated inhibition of apoptosis with delay of G2/M transition after DNA damage: a mechanism of resistance to multiple anticancer agents. *Blood.* 1995;86(3):1148-1158.
73. Amarante-Mendes GP, Naekyung Kim C, Liu L, et al. Bcr-Abl Exerts Its Antiapoptotic Effect Against Diverse Apoptotic Stimuli Through Blockage of Mitochondrial Release of Cytochrome C and Activation of Caspase-3. *Blood.* 1998;91(5):1700-1705.
74. Dubrez L, Eymin B, Sordet O, Droin N, Turhan AG, Solary E. BCR-ABL Delays Apoptosis Upstream of Procaspase-3 Activation. *Blood.* 1998;91(7):2415-2422.
75. Sánchez-Garcla I, MartIn-Zanca D. Regulation of Bcl-2 gene expression by BCR-ABL is mediated by Ras. *Journal of Molecular Biology.* 1997;267(2):225-228.
76. Neshat MS, Raitano AB, Wang H-G, Reed JC, Sawyers CL. The Survival Function of the Bcr-Abl Oncogene Is Mediated by Bad-Dependent and -Independent Pathways: Roles for Phosphatidylinositol 3-Kinase and Raf. *Molecular cell Biology.* 2000;20(4):1179-1186.
77. Brazil DP, Yang Z-Z, Hemmings BA. Advances in protein kinase B signalling: AKTion on multiple fronts. *Trends in Biochemical Sciences.* 2004;29(5):233-242.

78. Kharas MG, Fruman DA. ABL Oncogenes and Phosphoinositide 3-Kinase: Mechanism of Activation and Downstream Effectors. *Cancer Research*. 2005;65(6):2047-2053.
79. Oda T, Heaney C, Hagopian JR, Okuda K, Griffin JD, BJ. D. Crkl is the major tyrosine phosphorylated protein in neutrophils from patients with chronic myelogenous leukemia. *Journal of Biological Chemistry*. 1994;269: 22925-22928.
80. Sattler M, Salgia R, Okuda K, et al. The proto-oncogene product p120CBL and the adaptor proteins CRKL and c-CRK link c-ABL, p190BCR/ABL and p210BCR/ABL to the phosphatidylinositol-3' kinase pathway. *Oncogene*. 1996;12(4):839-846.
81. Feller S. Crk family adaptors-signalling complex formation and biological roles *Oncogene*. 2001;20(44):6348-6371.
82. Melo JV, Barnes DJ. Chronic myeloid leukaemia as a model of disease evolution in human cancer. *Nature Reviews Cancer*. 2007;7(6):441-453.
83. Roche-Lestienne C, Deluche L, Corm S, et al. RUNX1 DNA-binding mutations and RUNX1-PRDM16 cryptic fusions in BCR-ABL+ leukemias are frequently associated with secondary trisomy 21 and may contribute to clonal evolution and imatinib resistance. *Blood*. 2008;111(7):3735-3741.
84. Johansson B, Fioretos T, Mitelman F. Cytogenetic and Molecular Genetic Evolution of Chronic Myeloid Leukemia. *Acta Haematologica*. 2002;107(2):76-94.
85. Huntly BJP, Bench A, Green AR. Double jeopardy from a single translocation: deletions of the derivative chromosome 9 in chronic myeloid leukemia. *Blood*. 2003;102(4):1160-1168.
86. Herná J-C, Ndez b, Cervantes F, Costa D, Carrló A, Montserrat E. Blast Crisis of Ph-Positive Chronic Myeloid Leukemia with Isochromosome 17q: Report of 12 Cases and Review of the Literature. *Leukemia & Lymphoma*. 2000;38(1-2):83-90.
87. Nowicki MO, Falinski R, Koptyra M, et al. BCR/ABL oncogenic kinase promotes unfaithful repair of the reactive oxygen species–dependent DNA double-strand breaks. *Blood*. 2004;104(12):3746-3753.
88. Koptyra M, Falinski R, Nowicki MO, et al. BCR/ABL kinase induces self-mutagenesis via reactive oxygen species to encode imatinib resistance. *Blood*. 2006;108(1):319-327.
89. Eilers M, Eisenman RN. Myc's broad reach. *Genes & Development*. 2008;22(20):2755-2766.
90. Dang CV, O'Donnell KA, Zeller KI, Nguyen T, Osthus RC, Li F. The c-Myc target gene network. *Seminars in Cancer Biology*. 2006;16(4):253-264.
91. Khanna A, Bockelman C, Hemmes A, et al. MYC-dependent regulation and prognostic role of CIP2A in gastric cancer. *Journal of the National Cancer Institute*. 2009;101(11):793-805.
92. Wang J, Kim J, Roh M, et al. Pim1 kinase synergizes with c-MYC to induce advanced prostate carcinoma. *Oncogene*. 2010;29(17):2477-2487.
93. Côme C, Laine A, Chanrion M, et al. CIP2A Is Associated with Human Breast Cancer Aggressivity. *Clinical Cancer Research*. 2009;15(16):5092-5100.
94. Lucas CM, Harris RJ, Giannoudis A, Copland M, Slupsky JR, Clark RE. Cancerous inhibitor of PP2A (CIP2A) at diagnosis of chronic myeloid leukaemia is a critical determinant of disease progression. *Blood*. 2011;117(24):6660-6668.
95. Albajar M, Gómez-Casares MT, Llorca J, et al. MYC in Chronic Myeloid Leukemia: Induction of Aberrant DNA Synthesis and Association with Poor Response to Imatinib. *Molecular Cancer Research*. 2011;9(5):564-576.
96. Prochownik EV, Li Y. The ever expanding role for c-Myc in promoting genomic instability. *Cell Cycle*. 2007;6(9):1024-1029.

97. Oudat R, Khan Z, Glassman AB. Detection of Trisomy 8 in Philadelphia Chromosome-Positive CML Patients Using Conventional Cytogenetic and Interphase Fluorescence in situ Hybridization Techniques and its Relation to c-myc Involvement. *Annals of Clinical and Laboratory Science*. 2001;31(1):68-74.
98. Sawyers CL, Callahan W, Witte ON. Dominant negative MYC blocks transformation by ABL oncogenes. *Cell*. 1992;70(6):901-910.
99. Strnad M, Brajusković G, Strelić N, Zivanović BT, Tukić L, D. S. Expression of Bcl-2 protein and the amplification of c-myc gene in patients with chronic myeloid leukemia. *Vojnosanit Pregl*. 2006;63(4):364-369.
100. Dierov J, Dierova R, Carroll M. BCR/ABL translocates to the nucleus and disrupts an ATR-dependent intra-S phase checkpoint. *Cancer Cell*. 2004;5(3):275-285.
101. Bartek J, Lukas J. Chk1 and Chk2 kinases in checkpoint control and cancer. *Cancer Cell*. 2003;3(5):421-429.
102. Costanzo V, Shechter D, Lupardus PJ, Cimprich KA, Gottesman M, Gautier J. An ATR- and Cdc7-Dependent DNA Damage Checkpoint that Inhibits Initiation of DNA Replication. *Molecular Cell*. 2003;11(1):203-213.
103. Dierov JK, Schoppy DW, Carroll M. CML progenitor cells have chromsomal instability and display increased DNA damage at DNA fragile sites. *Blood*. 2005;106:563A.
104. Gordon MY, Dowding CR, Riley GP, Goldman JM, Greaves MF. Altered adhesive interactions with marrow stroma of haematopoietic progenitor cells in chronic myeloid leukaemia. *Nature*. 1987;328(6128):342-344.
105. Zhao RC, Tarone G, Verfaillie CM. Presence of the adhesion inhibitory β1B integrin isoform on CML but not normal progenitors is at least in part responsible for the decreased CML progenitor adhesion. *Blood*. 1997;90:393A.
106. Deininger MW. BCR-ABL tyrosine kinase activity regulates the expression of multiple genes implicated in the pathogenesis of chronic myeloid leukemia. *Cancer Research*. 2000;60:2049-2055.
107. Pelletier SD, Hong DS, Hu Y, Liu Y, Li S. Lack of the adhesion molecules P-selectin and intercellular adhesion molecule-1 accelerate the development of BCR/ABL-induced chronic myeloid leukemia-like myeloproliferative disease in mice. *Blood*. 2004;104(7):2163-2171.
108. Perrotti D, Cesi V, Trotta R, et al. BCR-ABL suppresses C/EBP[alpha] expression through inhibitory action of hnRNP E2. *Nature Genetics*. 2002;30(1):48-58.
109. Mullighan CG, Miller CB, Radtke I, et al. BCR-ABL1 lymphoblastic leukaemia is characterized by the deletion of Ikaros. *Nature*. 2008;453(7191):110-114.
110. Kirstetter P, Thomas M, Dierich A, Kastner P, Chan S. Ikaros is critical for B cell differentiation and function. *European Journal of Immunology*. 2002;32(3):720-730.
111. Baccarani M, Cortes J, Pane F, et al. Chronic Myeloid Leukemia: An Update of Concepts and Management Recommendations of European LeukemiaNet *Journal of Clinical Oncology*. 2009;27(35):6041-6051.
112. Wang L, Pearson K, Pillitteri L, Ferguson JE, Clark RE. Serial monitoring of BCR-ABL by peripheral blood real-time polymerase chain reaction predicts the marrow cytogenetic response to imatinib mesylate in chronic myeloid leukaemia. *British Journal of Haematology*. 2002;118(3):771-777.
113. Hughes T, Deininger M, Hochhaus A, et al. Monitoring CML patients responding to treatment with tyrosine kinase inhibitors: review and recommendations for harmonizing current methodology for detecting BCR-ABL transcripts and kinase domain mutations and for expressing results. *Blood*. 2006;108(1):28-37.

114. Hehlmann R, Heimpel H, Hasford J, et al. Randomized comparison of interferon-alpha with busulfan and hydroxyurea in chronic myelogenous leukemia. The German CML Study Group. *Blood.* 1994;84(12):4064-4077.
115. Dowding C, Gordon M, Guo A, et al. Potential mechanisms of action of interferon-alpha in CML. *Leukaemia and Lymphoma* 1993;11 (Supplement 1):185-191.
116. Allan NC, Shepherd PCA, Richards SM. UK Medical Research Council randomised, multicentre trial of interferon-[alpha]n1 for chronic myeloid leukaemia: improved survival irrespective of cytogenetic response. *The Lancet.* 1995;345(8962):1392-1397.
117. Ozer H, George SL, Schiffer CA, et al. Prolonged subcutaneous administration of recombinant alpha 2b interferon in patients with previously untreated Philadelphia chromosome-positive chronic-phase chronic myelogenous leukemia: effect on remission duration and survival: Cancer and Leukemia Group B study 8583. *Blood.* 1993;82(10):2975-2984.
118. Goldman JM, Baughan AS, McCarthy DM, et al. Marrow transplantation for patients in the chronic phase of chronic granulocytic leukaemia. *Lancet.* 1982;2(8299):623-625.
119. Fefer A, Cheever MA, Thomas ED, et al. Disappearance of pH1-Positive Cells in Four Patients with Chronic Granulocytic Leukemia after Chemotherapy, Irradiation and Marrow Transplantation from an Identical Twin. *New England Journal of Medicine.* 1979;300(7):333-337.
120. Hehlmann R, Berger U, Pfirrmann M, et al. Drug treatment is superior to allografting as first-line therapy in chronic myeloid leukemia. *Blood.* 2007;109:4686-4692.
121. Silver RT, Woolf SH, Hehlmann R, et al. An Evidence-Based Analysis of the Effect of Busulfan, Hydroxyurea, Interferon, and Allogeneic Bone Marrow Transplantation in Treating the Chronic Phase of Chronic Myeloid Leukemia: Developed for the American Society of Hematology. *Blood.* 1999;94(5):1517-1536.
122. Italian Cooperative Study Group on Chronic Myeloid Leukemia and Italian Group for Bone Marrow Transplantation. Monitoring Treatment and Survival in Chronic Myeloid Leukemia. *Journal of Clinical Oncology.* 1999;17(6):1858-1868.
123. Hehlmann R, Hochhaus A, Baccarani M. Chronic myeloid leukaemia. *The Lancet.* 2007;370(9584):342-350.
124. Gratwohl A, Hermans J, Goldman JM, et al. Risk assessment for patients with chronic myeloid leukaemia before allogeneic blood or marrow transplantation. *The Lancet.* 1998;352(9134):1087-1092.
125. Anafi M, Gazit A, Zehavi A, Ben-Neriah Y, Levitzki A. Tyrphostin-induced inhibition of p210bcr-abl tyrosine kinase activity induces K562 to differentiate. *Blood.* 1993;82(12):3524-3529.
126. Kaur G, Gazit A, Levitzki A, Stowe E, Cooney DA, Sausville EA. Tyrphostin induced growth inhibition: correlation with effect on p210bcr-abl autokinase activity in K562 chronic myelogenous leukemia. *Anticancer Drugs.* 1994;5(2):213-222.
127. Shiotsu Y, Neckers LM, Wortman I, et al. Novel oxime derivatives of radicicol induce erythroid differentiation associated with preferential G1 phase accumulation against chronic myelogenous leukemia cells through destabilization of Bcr-Abl with Hsp90 complex. *Blood.* 2000;96(6):2284-2291.
128. Zimmermann J, Caravatti G, Mett H, et al. Phenylamino-pyrimidine (PAP) derivatives: a new class of potent and selective inhibitors of protein kinase C (PKC). *Arch Pharm (Weinheim).* 1996;329(7):371-376.
129. Zimmermann J, Buchdunger E, Mett H, Meyer T, Lydon NB, Traxler P. Phenylamino-pyrimidine (PAP) -- derivatives: a new class of potent and highly selective PDGF-receptor autophosphorylation inhibitors. *Bioorganic & Medicinal Chemistry Letters.* 1996;6(11):1221-1226.

130. Zimmermann J, Buchdunger E, Mett H, Meyer T, Lydon NB. Potent and selective inhibitors of the Abl-kinase: phenylamino-pyrimidine (PAP) derivatives. *Bioorganic & Medicinal Chemistry Letters*. 1997;7(2):187-192.
131. Hughes TP, Kaeda J, Branford S, et al. Frequency of Major Molecular Responses to Imatinib or Interferon Alfa plus Cytarabine in Newly Diagnosed Chronic Myeloid Leukemia. *New England Journal of Medicine*. 2003;349(15):1423-1432.
132. Druker BJ, Talpaz M, Resta DJ, Peng B, Buchdunger E, Ford JM. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *New England Journal of Medicine*. 2001;344:1031-1037.
133. Guilhot F, Chastang C, Michallet M, et al. Interferon Alfa-2b Combined with Cytarabine versus Interferon Alone in Chronic Myelogenous Leukemia. *New England Journal of Medicine*. 1997;337(4):223-229.
134. Kerkela R, Grazette L, Yacobi R, et al. Cardiotoxicity of the cancer therapeutic agent imatinib mesylate. *Nature Medicine*. 2006;12(8):908-916.
135. Wolf A, Couttet P, Dong M, et al. Imatinib does not induce cardiotoxicity at clinically relevant concentrations in preclinical studies. *Leukemia Research*. 2010;34(9):1180-1188.
136. Estabragh ZR, Knight K, Watmough SJ, et al. A prospective evaluation of cardiac function in patients with chronic myeloid leukaemia treated with imatinib. *Leukemia Research*. 2011;35(1):49-51.
137. Kantarjian HM, Talpaz M, O'Brien S, et al. Dose escalation of imatinib mesylate can overcome resistance to standard-dose therapy in patients with chronic myelogenous leukemia. *Blood*. 2003;101(2):473-475.
138. Hughes TP, Branford S, White DL, et al. Impact of early dose intensity on cytogenetic and molecular responses in chronic- phase CML patients receiving 600 mg/day of imatinib as initial therapy. *Blood*. 2008;112(10):3965-3973.
139. Preudhomme C, Guilhot J, Nicolini FE, et al. Imatinib plus Peginterferon Alfa-2a in Chronic Myeloid Leukemia. *New England Journal of Medicine*. 2010;363(26):2511-2521.
140. Hehlmann R, Lauseker M, Jung-Munkwitz S, et al. Tolerability-Adapted Imatinib 800 mg/d Versus 400 mg/d Versus 400 mg/d Plus Interferon-α in Newly Diagnosed Chronic Myeloid Leukemia. *Journal of Clinical Oncology*. 2011;29(12):1634-1642.
141. Hughes T, Hochhaus A. Clinical strategies to achieve an early and successful response to a tyrosine kinase inhibitor therapy. *Seminars in Hematology*. 2009;46(2, supplement 3):11-15.
142. Shah NP, Tran C, Lee FY, Chen P, Norris D, Sawyers CL. Overriding Imatinib Resistance with a Novel ABL Kinase Inhibitor. *Science*. 2004;305(5682):399-401.
143. Tokarski JS, Newitt JA, Chang CYJ, et al. The Structure of Dasatinib (BMS-354825) Bound to Activated ABL Kinase Domain Elucidates Its Inhibitory Activity against Imatinib-Resistant ABL Mutants. *Cancer Research*. 2006;66(11):5790-5797.
144. Talpaz M, Shah NP, Kantarjian H, et al. Dasatinib in Imatinib-Resistant Philadelphia Chromosome-Positive Leukemias. *New England Journal of Medicine*. 2006;354(24):2531-2541.
145. Hochhaus A, Kantarjian HM, Baccarani M, et al. Dasatinib induces notable hematologic and cytogenetic responses in chronic-phase chronic myeloid leukemia after failure of imatinib therapy. *Blood*. 2007;109(6):2303-2309.
146. Kantarjian H, Shah NP, Hochhaus A, et al. Dasatinib versus Imatinib in Newly Diagnosed Chronic-Phase Chronic Myeloid Leukemia. *New England Journal of Medicine*. 2010;362(24):2260-2270.
147. Giannoudis A, Davies A, Lucas CM, Harris RJ, Pirmohamed M, Clark RE. Effective dasatinib uptake may occur without human organic cation transporter 1 (hOCT1):

implications for the treatment of imatinib-resistant chronic myeloid leukemia. *Blood.* 2008;112(8):3348-3354.
148. Kantarjian HM, Giles F, Gattermann N, et al. Nilotinib (formerly AMN107), a highly selective Bcr-Abl tyrosine kinase inhibitor, is effective in patients with Philadelphia chromosome-positive chronic myelogenous leukemia in chronic phase following imatinib resistance and intolerance. *Blood.* 2007;110:3540-3546.
149. Rix U, Hantschel O, Durnberger G, et al. Chemical proteomic profiles of the BCR-ABL inhibitors imatinib, nilotinib and dasatinib reveal novel kinase and non-kinase targets. *Blood.* 2007;110(12):4055-4063.
150. Kantarjian HM, Giles FJ, Bhalla KN, et al. Nilotinib is effective in patients with chronic myeloid leukemia in chronic phase after imatinib resistance or intolerance: 24-month follow-up results. *Blood.* 2011;117(4):1141-1145.
151. Hochhaus A, Saglio G, le Coutre P, et al. Superior efficacy of nilotinib compared with imatinib in newly-diagnosed patients with chronic myeloid leukemia in chronic (CML-CP): ENESTnd minimum 24-month follow-up. *Haematologica.* 2011;96(Supplement 2):484A.
152. Saglio G, Kim D-W, Issaragrisil S, et al. Nilotinib versus Imatinib for Newly Diagnosed Chronic Myeloid Leukemia. *New England Journal of Medicine.* 2010;362(24):2251-2259.
153. Radich JP. Optimizing timing of secondary tyrosine kinase therapy in chronic myeloid leukemia. *Clinical Lymphoma and Myeloma* 2008;Supplement 3:S89-94.
154. Shah NP. Advanced CML: therapeutic options for patients in accelerated and blast phases. *Journal National Comprehensive Cancer Network* 2008;Supplement 2:S31-S36.
155. Sokal JE, Cox EB, Baccarani M, et al. Prognostic discrimination in "good-risk" chronic granulocytic leukemia. *Blood.* 1984;63(4):789-799.
156. Sokal JE, Baccarani M, Tura S. Prognostic discrimination among younger patients with chronic granulocytic leukemia: Relevance to bone marrow transplantation. *Blood.* 1985;66(6):1352-1357.
157. Sokal JE, Gomez GA, Baccarani M, et al. Prognostic significance of additional cytogenetic abnormalities at diagnosis of Philadelphia chromosome-positive chronic granulocytic leukemia. *Blood.* 1988;72(1):294-298.
158. Hasford J, Pfirrmann M, Hehlmann R, et al. A New Prognostic Score for Survival of Patients With Chronic Myeloid Leukemia Treated With Interferon AlfaWriting Committee for the Collaborative CML Prognostic Factors Project Group. *Journal of the National Cancer Institute.* 1998;90(11):850-859.
159. O'Brien SG, Guilhot F, Goldman JM, et al. International Randomized Study of Interferon Versus STI571 (IRIS) 7-Year Follow-up: Sustained Survival, Low Rate of Transformation and Increased Rate of Major Molecular Response (MMR) in Patients (pts) with Newly Diagnosed Chronic Myeloid Leukemia in Chronic Phase (CMLCP) Treated with Imatinib (IM). *Blood.* 2008;112(11):Abstract 186.
160. Hasford J, Baccarani M, Hoffmann V, et al. Predicting complete cytogenetic response and subsequent progression-free survival in 2060 patients with CML on imatinib treatment: the EUTOS score. *Blood.* 2011;118(3):686-692.
161. Huntly BJP, Reid AG, Bench AJ, et al. Deletions of the derivative chromosome 9 occur at the time of the Philadelphia translocation and provide a powerful and independent prognostic indicator in chronic myeloid leukemia. *Blood.* 2001;98(6):1732-1738.
162. Huntly BJP, Guilhot F, Reid AG, et al. Imatinib improves but may not fully reverse the poor prognosis of patients with CML with derivative chromosome 9 deletions. *Blood.* 2003;102(6):2205-2212.

163. Wang L, Pearson K, Ferguson JE, Clark RE. The early molecular response to imatinib predicts cytogenetic and clinical outcome in chronic myeloid leukaemia. *British Journal of Haematology*. 2003;120:990-999.
164. Hughes TP, Hochhaus A, Branford S, et al. Long-term prognostic significance of early molecular response to imatinib in newly diagnosed chronic myeloid leukemia: an analysis from the International Randomized Study of Interferon and STI571 (IRIS). *Blood*. 2010;116(19):3758-3765.
165. Thomas J, Wang L, Clark RE, Pirmohamed M. Active transport of imatinib into and out of cells: implications for drug resistance. *Blood*..2004;12 (104):3739-3745.
166. Wang L, Giannoudis A, Lane S, Williamson P, Pirmohamed M, Clark RE. Expression of the Uptake Drug Transporter hOCT1 is an Important Clinical Determinant of the Response to Imatinib in Chronic Myeloid Leukemia. *Clinical Pharmacology and Therapeutics*. 2008;83(2):258-264.
167. White DL, Saunders VA, Dang P, et al. OCT-1-mediated influx is a key determinant of the intracellular uptake of imatinib but not nilotinib (AMN107): reduced OCT-1 activity is the cause of low in vitro sensitivity to imatinib. *Blood*. 2006;108:697-704.
168. Yamada O, Ozaki K, Furukawa T, et al. Activation of STAT5 confers imatinib resistance on leukemic cells through the transcription of TERT and MDR1. *Cellular Signalling*. 2011;23(7):1119-1127.
169. Velingkar VS, Dandekar VD. Modulation of p-glycoprotein mediated multidrug resistance (MDR) in cancer using chemosensitizer. *International Journal of Pharma Sciences and Research (IJPSR)*. 2010;1(2):104-111.
170. Illmer T. P-glycoprotein-mediated drug efflux is a resistance mechanism of chronic myelogenous leukemia cells to treatment with imatinib mesylate. *Leukemia*. 2004;18:401-408.
171. Burger H. Chronic imatinib mesylate exposure leads to reduced intracellular drug accumulation by induction of the ABCG2 (BCRP) and ABCB1 (MDR1) drug transport pumps. *Cancer Biology and Therapy*. 2005;4:747-752.
172. Dulucq S, Bouchet S, Turcq B, et al. Multidrug resistance gene (MDR1) polymorphisms are associated with major molecular responses to standard-dose imatinib in chronic myeloid leukemia. *Blood*. 2008;112(5):2024-2027.
173. Picard S, Titier K, Etienne G, et al. Trough imatinib plasma levels are associated with both cytogenetic and molecular responses to standard-dose imatinib in chronic myeloid leukemia. *Blood*. 2007;109(8):3496-3499.
174. Larson RA, Druker BJ, Guilhot F, et al. Imatinib pharmacokinetics and its correlation with response and safety in chronic-phase chronic myeloid leukemia: a subanalysis of the IRIS study. *Blood*. 2008;111(8):4022-4028.
175. Davies A, Hayes AK, Giannoudis A, et al. A study of plasma levels of imatinib and its bioactive metabolite CGP-74588 reveals no correlation with subsequent clinical outcome in imatinib-treated chronic myeloid leukaemia. *Haematologica*. 2009;94 S2(Abstract 637):258.
176. Forrest DL, Trainor S, Brinkman RR, et al. Cytogenetic and molecular responses to standard-dose imatinib in chronic myeloid leukemia are correlated with Sokal risk scores and duration of therapy but not trough imatinib plasma levels. *Leukemia Research*. 2009;33(2):271-275.
177. Guilhot F, Apperley J, Kim D, et al. Dasatinib induces significant hematologic and cytogenetic responses in patients with imatinib-resistant or -intolerant chronic myeloid leukemia in accelerated phase. *Blood*. 2007;109(10):4143-4150.
178. Deininger M, Buchdunger E, Druker BJ. The development of imatinib as a therapeutic agent for chronic myeloid leukemia. *Blood*. 2005;105(7):2640-2653.

179. Willis SG, Lange T, Demehri S, et al. High-sensitivity detection of BCR-ABL kinase domain mutations in imatinib-naive patients: correlation with clonal cytogenetic evolution but not response to therapy. *Blood.* 2005;106(6):2128-2137.
180. Jabbour E, Soverini S. Understanding the role of mutations in therapeutic decision making for chronic myeloid leukemia. *Seminars in Hematology.* 2009;46(2 supplement 3):s22-26.
181. Gorre ME. Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. *Science.* 2001;293:876-880.
182. Nicolini FE, Corm S, Le QH, et al. Mutation status and clinical outcome of 89 imatinib mesylate-resistant chronic myelogenous leukemia patients: a retrospective analysis from the French intergroup of CML (Fi([phiv])-LMC GROUP). *Leukemia.* 2006;20(6):1061-1066.
183. O'Hare T, Eide CA, Deininger MWN. Bcr-Abl kinase domain mutations, drug resistance, and the road to a cure for chronic myeloid leukemia. *Blood.* 2007;110(7):2242-2249.
184. Soverini S, Martinelli G, Rosti G, et al. ABL mutations in late chronic phase chronic myeloid leukemia patients with up-front cytogenetic resistance to imatinib are associated with a greater likelihood of progression to blast crisis and shorter survival: a study by the GIMEMA Working Party on Chronic Myeloid Leukemia. *Journal Clinical Oncology.* 2005;23:4100-4109.
185. Kantarjian H, Pasquini R, Hamerschlak N, et al. Dasatinib or high-dose imatinib for chronic-phase chronic myeloid leukemia after failure of first-line imatinib: a randomized phase 2 trial. *Blood.* 2007;109(12):5143-5150.
186. Bengió RM, Riva ME, Moiraghi B, et al. Clinical outcome of chronic myeloid leukemia imatinib-resistant patients: do BCR–ABL kinase domain mutations affect patient survival? First multicenter Argentinean study. *Leukemia & Lymphoma.* 2011;Epub ahead of print.
187. Branford S. Detection of BCR-ABL mutations in patients with CML treated with imatinib is virtually always accompanied by clinical resistance, and mutations in the ATP phosphate-binding loop (P-loop) are associated with a poor prognosis. *Blood.* 2003;102:276-283.
188. Wang L, Knight K, Lucas C, Clark RE. The role of serial BCR-ABL transcript monitoring in predicting the emergence of BCR-ABL kinase mutations in imatinib-treated patients with chronic myeloid leukemia. *Haematologica.* 2006;91(2):235-239.
189. Patel H, Marley SB, Gordon MY. Conventional Western blotting techniques will not reliably quantify p210BCR-ABL1 levels in CML mononuclear cells. *Blood.* 2007;109(3):1335.
190. ten Hoeve J, Arlinghaus RB, Guo JQ, Heisterkamp N, Groffen J. Tyrosine phosphorylation of CRKL in Philadelphia+ leukemia. . *Blood.* 1994;84 1731-1736.
191. Senechal K, Halpern J, Sawyers CL. The CRKL adaptor protein transforms fibroblasts and functions in transformation by the BCR-ABL oncogene. *Journal of Biological Chemistry.* 1996;271(38):23255-23261.
192. ten Hoeve J, Morris C, Heisterkamp N, Groffen J. Isolation and chromosomal localization of CRKL, a human crk-like gene. *Oncogene* 1993;8:2469-2474.
193. Sattler M, R. S. Role of the adapter protein CRKL in signal transduction of normal hematopoietic and BCR/ABL-transformed cells. *Leukemia* 1998;12(5):637-644.
194. Sattler M, Salgia R, Shrikhande G, et al. Differential Signaling after beta 1 Integrin Ligation Is Mediated Through Binding of CRKL to p120CBL and p110HEF1. *Journal of Biological Chemistry.* 1997;272(22):14320-14326.

195. Feller S, Posern G, Voss J, et al. Physiological signals and oncogenesis mediated through Crk family adapter proteins. *Journal of Cell Physiology*. 1998;177:535-552.
196. Hemmeryckx B, van Wijk A, Reichert A, et al. Crkl Enhances Leukemogenesis in BCR/ABL P190 Transgenic Mice. *Cancer Research* 2001;61:398-1405.
197. White DL, Saunders V, Lyons AB, et al. In vitro sensitivity to imatinib-induced inhibition of ABL kinase activity is predictive of molecular response in patients with de novo CML. *Blood*. 2005;106(7):2520-2526.
198. White DL, Saunders V, Grigg A, et al. Measurement of In Vivo BCR-ABL Kinase Inhibition to Monitor Imatinib-Induced Target Blockade and Predict Response in Chronic Myeloid Leukemia. *Journal of Clinical Oncology*. 2007;25(28):4445-4451.
199. Hamilton A, Elrick L, Myssina S, et al. BCR-ABL activity and its response to drugs can be determined in CD34+ CML stem cells by CrkL phosphorylation status using flow cytometry. *Leukemia*. 2006;20:1035-1039.
200. Singer CF, Hudelist G, Lamm W, et al. Active (p)CrkL is overexpressed in human malignancies: potential role as a surrogate parameter for therapeutic tyrosine kinase inhibition. *Oncology Reports*. 2006;15:353-359.
201. Kim YH, Kwei KA, Girard L, et al. Genomic and functional analysis identifies CRKL as an oncogene amplified in lung cancer. *Oncogene*. 2010;29(10):1421-1430.
202. Moon AM, Guris DL, Seo J, et al. Crkl Deficiency Disrupts Fgf8 Signaling in a Mouse Model of 22q11 Deletion Syndromes. *Cell*. 2006;10(1):71-80.
203. Khorashad JS, Wagner S, Greener L, et al. The level of BCR-ABL1 kinase activity before treatment does not identify chronic myeloid leukemia patients who fail to achieve a complete cytogenetic response on imatinib. *Haematologica*. 2009;94(6):861-864.
204. Perrotti D, Jamieson C, Goldman J, Skorski T. Chronic myeloid leukemia: mechanisms of blastic transformation. *The Journal of Clinical Investigation*. 2010;120(7):2254-2264.
205. Oehler VG, Yeung KY, Choi YE, Bumgarner RE, Raftery AE, Radich JP. The derivation of diagnostic markers of chronic myeloid leukemia progression from microarray data. *Blood*. 2009;114(15):3292-3298.
206. Yong ASM, Szydlo RM, Goldman JM, Apperley JF, Melo JV. Molecular profiling of CD34+ cells identifies low expression of CD7, along with high expression of proteinase 3 or elastase, as predictors of longer survival in patients with CML. *Blood*. 2006;107(1):205-212.
207. Lanza F, Bi S. Role of p53 in leukemogenesis of chronic myeloid leukemia. *Stem Cells*. 1995;13(4):445-452.
208. Peterson LF, Mitrikeska E, Giannola D, et al. p53 stabilization induces apoptosis in chronic myeloid leukemia blast crisis cells. *Leukemia*. 2011;25(5):761-769.
209. Ahuja H, Bar-Eli M, Advani SH, Benchimol S, Cline MJ. Alterations in the p53 gene and the clonal evolution of the blast crisis of chronic myelocytic leukemia. *PNAS*. 1989;86(17):6783-6787.
210. Nakayama H, Ishimaru F, Avitahl N, et al. Decreases in Ikaros Activity Correlate with Blast Crisis in Patients with Chronic Myelogenous Leukemia. *Cancer Research*. 1999;59(16):3931-3934.
211. Mullighan CG, Williams RT, Downing JR, Sherr CJ. Failure of CDKN2A/B (INK4A/B-ARF)-mediated tumor suppression and resistance to targeted therapy in acute lymphoblastic leukemia induced by BCR-ABL. *Genes and Development*. 2008;22(11):1411–1415.
212. Daghistani M, Marin D, Khorashad JS, et al. EVI-1 oncogene expression predicts survival in chronic-phase CML patients resistant to imatinib treated with second-generation tyrosine kinase inhibitors. *Blood*. 2010;116(26):6014-6017.

213. Varma N, Anand MS, Varma S, Juneja SS. Role of hTERT and WT1 gene expression in disease progression and imatinib responsiveness of patients with BCR–ABL positive chronic myeloid leukemia. *Leukemia & Lymphoma*. 2011;52(4):687-693.
214. Venturini L, Battmer K, Castoldi M, et al. Expression of the miR-17-92 polycistron in chronic myeloid leukemia (CML) CD34+ cells. *Blood*. 2007;109(10):4399-4405.
215. Porro A, Iraci N, Soverini S, et al. c-MYC oncoprotein dictates transcriptional profiles of ATP-binding cassette transporter genes in Chronic Myelogenous Leukemia CD34+ hematopoietic progenitor cells. *Molecular Cancer Research*. 2011.
216. Samanta AK, Chakraborty SN, Wang Y, et al. Jak2 inhibition deactivates Lyn kinase through the SET-PP2A-SHP1 pathway, causing apoptosis in drug-resistant cells from chronic myelogenous leukemia patients. *Oncogene*. 2009;28(14):1669-1681.
217. Wu J, Meng F, Lu H, et al. Lyn regulates BCR-ABL and Gab2 tyrosine phosphorylation and c-Cbl protein stability in imatinib-resistant chronic myelogenous leukemia cells. *Blood*. 2008;111(7):3821-3829.
218. Eiring AM, Harb JG, Neviani P, et al. miR-328 functions as an RNA decoy to modulate hnRNP E2 regulation of mRNA translation in leukemic blasts. *Cell*. 2010;140(5):652-665.
219. Scherr M, Chaturvedi A, Battmer K, et al. Enhanced sensitivity to inhibition of SHP2, STAT5, and Gab2 expression in chronic myeloid leukemia (CML). *Blood*. 2006;107(8):3279-3287.
220. Bhattacharyya J, Mihara K, Yasunaga Si, et al. BMI-1 expression is enhanced through transcriptional and posttranscriptional regulation during the progression of chronic myeloid leukemia. *Annals of Hematology*. 2009;88(4):333-340.
221. Puisieux A, Valsesia-Wittmann S, Ansieau S. A twist for survival and cancer progression. *British Journal of Cancer*. 2005;94(1):13-17.
222. Valsesia-Wittmann S, Magdeleine M, Dupasquier S, et al. Oncogenic cooperation between H-Twist and N-Myc overrides failsafe programs in cancer cells. *Cancer Cell*. 2004;6(6):625-630.
223. Cosset E, Hamdan G, Jeanpierre S, et al. Deregulation of TWIST-1 in the CD34+ compartment represents a novel prognostic factor in chronic myeloid leukemia. *Blood*. 2011;117(5):1673-1676.
224. Jordan CT, Guzman ML, Noble M. Cancer Stem Cells. *New England Journal of Medicine*. 2006;355(12):1253-1261.
225. Wang JCY, Dick JE. Cancer stem cells: lessons from leukemia. *Trends in Cell Biology*. 2005;15(9):494-501.
226. Chen Y, Li D, Li S. The Alox5 gene is a novel therapeutic target in cancer stem cells of chronic myeloid leukemia. *Cell Cycle*. 2009;8(21):3488-3492.
227. Barker N, Clevers H. Catenins, Wnt signaling and cancer. *BioEssays*. 2000;22(11):961-965.
228. Radich JP, Dai H, Mao M, et al. Gene expression changes associated with progression and response in chronic myeloid leukemia. *PNAS*. 2006;103(8):2794-2799.
229. MacDonald BT, Tamai K, He X. Wnt/[beta]-Catenin Signaling: Components, Mechanisms, and Diseases. *Developmental Cell*. 2009;17(1):9-26.
230. Athar M, Tang X, Lee JL, Kopelovich L, Kim AL. Hedgehog signalling in skin development and cancer. *Exp Dermatol*. 2006;15:667-677.
231. Sengupta A, Banerjee D, Chandra S, et al. Deregulation and cross talk among Sonic hedgehog, Wnt, Hox and Notch signaling in chronic myeloid leukemia progression. *Leukemia*. 2007;21(5):949-955.

232. Manev H, Manev R. 5-Lipoxygenase (ALOX5) and FLAP (ALOX5AP) gene polymorphisms as factors in vascular pathology and Alzheimer's disease. *Medical Hypotheses*. 2006;66(3):501-503.
233. Yokomizo T, Uozumi N, Takahashi T, Kume K, Izumi T, Shimizu T. Leukotriene A4 hydrolase and leukotriene B4 metabolism. *J Lipid Mediat Cell Signal*. 1995;12:321-332.
234. Drazen JM, Yandava CN, Dube L, et al. Pharmacogenetic association between ALOX5 promoter genotype and the response to anti-asthma treatment. *Nature Genetics*. 1999;22(2):168-170.
235. Dwyer JH, Allayee H, Dwyer KM, et al. Arachidonate 5-Lipoxygenase Promoter Genotype, Dietary Arachidonic Acid, and Atherosclerosis. *New England Journal of Medicine*. 2004;350(1):29-37.
236. Hoque A, Lippman SM, Wu T, et al. Increased 5-lipoxygenase expression and induction of apoptosis by its inhibitors in esophageal cancer: a potential target for prevention. *Carcinogenesis*. 2005;26(4):785-791.
237. Ohnishi H, Miyahara N, Gelfand EW. The Role of Leukotriene B4 in Allergic Diseases. *Allergology International*. 2008;4:291-298.
238. Choi JA, Kim EY, Song H, Kim C, Kim JH. Reactive oxygen species are generated through a BLT2-linked cascade in Ras-transformed cells. *Free Radical Biology and Medicine*. 2008;44:624-634.
239. Tager AM, Luster AD. BLT1 and BLT2: the leukotriene B4 receptors. *Prostaglandins, Leukotrienes and Essential Fatty Acids*. 2003;69(2-3):123-134.
240. Luster AD, Tager AM. T-cell trafficking in asthma: lipid mediators grease the way. *Nature Reviews Immunolology*. 2004;4(9):711-724.
241. Hicks A, Monkarsh SP, Hoffman AF, Goodnow R. Leukotriene B4 receptor antagonists as therapeutics for inflammatory disease: preclinical and clinical developments. *Expert Opinion on Investigational Drugs*. 2007;16(12):1909-1920.
242. Kalayci O, Birben E, Sackesen C, et al. ALOX5 promoter genotype, asthma severity and LTC production by eosinophils. *Allergy*. 2006;61(1):97-103.
243. Telleria JJ, Blanco-Quiros A, Varillas D, et al. ALOX5 promoter genotype and response to montelukast in moderate persistent asthma. *Respirtory Medicine*. 2008;102(6):857-861.
244. Mastalerz L, Jagoda K. Antileukotriene drugs in the treatment of asthma. *Polish archieve of internal medicine*. 2010;120(3):103-108.
245. Sánchez-Galán E, Gómez-Hernández A, Vidal C, et al. Leukotriene B4 enhances the activity of nuclear factor-κB pathway through BLT1 and BLT2 receptors in atherosclerosis. *Cardiovascular Research*. 2009;81(1):216-225.
246. Ding XZ, Tong WG, Adrian TE. Multiple Signal Pathways Are Involved in the Mitogenic Effect of 5(S)-HETE in Human Pancreatic Cancer. *Oncology*. 2003;65(4):285-294.
247. Heukamp I, Kilian M, Gregor JI, Kiewert C, Schimke I, Kristiansen G. Impact of polyunsaturated fatty acids on hepato-pancreatic prostaglandin and leukotriene concentration in ductal pancreatic cancer -- is there a correlation to tumour growth and liver metastasis? *Prostaglandins Leukot Essent Fatty Acids*. 2006;74:223-233.
248. Ye YN, Wu WKK, Shin VY, Bruce IC, Wong BCY, Cho CH. Dual inhibition of 5-LOX and COX-2 suppresses colon cancer formation promoted by cigarette smoke. *Carcinogenesis*. 2005;26(4):827-834.
249. Goode EL, Maurer MJ, Sellers TA, et al. Inherited determinants of ovarian cancer survival. *Clinical Cancer Research*. 2010;16(3):995-1007.
250. Graham SM. Primitive, quiescent, Philadelphia-positive stem cells from patients with chronic myeloid leukemia are insensitive to STI571 in vitro. *Blood*. 2002;99:319-325.

251. Jørgensen HG, Allan EK, Jordanides NE, Mountford JC, Holyoake TL. Nilotinib exerts equipotent antiproliferative effects to imatinib and does not induce apoptosis in CD34+ CML cells. *Blood.* 2007;109(9):4016-4019.
252. Copland M, Pellicano F, Richmond L, et al. BMS-214662 potently induces apoptosis of chronic myeloid leukemia stem and progenitor cells and synergises with tyrosine kinase inhibitors. *Blood.* 2007;111(9):4830.
253. Chen Y, Hu Y, Zhang H, Peng C, Li S. Loss of the Alox5 gene impairs leukemia stem cells and prevents chronic myeloid leukemia. *Nature Genetics.* 2009;41(7):783-792.
254. Janssens V, Goris J. Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *Biochemical Journal.* 2001;353:417-439.
255. Wang Z, Shen D, Parsons DW, et al. Mutational analysis of the tyrosine phosphatome in colorectal cancers. *Science* 2004;304(5674):1164-1166.
256. Janssens V, Goris J, Van Hoof C. PP2A: the expected tumor suppressor. *Current Opinion in Genetics & Development.* 2005;15(1):34-41.
257. Eichhorn PJA, Creyghton MP, Bernards R. Protein phosphatase 2A regulatory subunits and cancer. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer.* 2009;1795(1):1-15.
258. Favre B, Zolnierowicz S, Turowski P, Hemmings BA. The catalytic subunit of protein phosphatase 2A is carboxyl-methylated in vivo. *Journal of Biological Chemistry.* 1994;269(23):16311-16317.
259. De Baere I, Derua R, Janssens V, et al. Purification of Porcine Brain Protein Phosphatase 2A Leucine Carboxyl Methyltransferase and Cloning of the Human Homologue. *Biochemistry.* 1999;38(50):16539-16547.
260. Zhu T, Matsuzawa S-i, Mizuno Y, et al. The interconversion of Protein Phosphatase 2A between PP2A1and PP2A during retinoic acid-induced granulocytic differentiation and a modification on the catalytic subunit in S phase of HL-60 cells. *Archives of Biochemistry and Biophysics.* 1997;339(1):210-217.
261. Yong ASM, Melo JV. The impact of gene profiling in chronic myeloid leukaemia. *Best Practice & Research Clinical Haematology.* 2009;22(2):181-190.
262. Francia G, Poulsom R, Hanby AM, et al. Identification by differential display of a protein phosphatase-2A regulatory subunit preferentially expressed in malignant melanoma cells. *International Journal of Cancer.* 1999;82(5):709-713.
263. Chen W, Possemato R, Campbell KT, Plattner CA, Pallas DC, Hahn WC. Identification of specific PP2A complexes involved in human cell transformation. *Cancer Cell.* 2004;5(2):127-136.
264. Neviani P, Santhanam R, Trotta R, et al. The tumor suppressor PP2A is functionally inactivated in blast crisis CML through the inhibitory activity of the BCR/ABL-regulated SET protein. *Cancer Cell.* 2005;8(5):355-368.
265. Cristobal I, Blanco FJ, Garcia-Orti L, et al. SETBP1 overexpression is a novel leukemogenic mechanism that predicts adverse outcome in elderly patients with acute myeloid leukemia. *Blood.* 2010;115(3):615-625.
266. Perrotti D, Neviani P. ReSETting PP2A tumour suppressor activity in blast crisis and imatinib-resistant chronic myelogenous leukaemia. *British Journal of Cancer.* 2006;95(7):775-781.
267. Adachi Y, Pavlakis G, Copeland T. Identification of in vivo phosphorylation sites of SET, a nuclear phosphoprotein encoded by the translocation breakpoint in acute undifferentiated leukemia. *FEBS Letters.* 1994;340(3):231-235.
268. Perrotti D, Neviani P. From mRNA Metabolism to Cancer Therapy: Chronic Myelogenous Leukemia Shows the Way. *Clinical Cancer Research.* 2007;13(6):1638-1642.

269. Carlson S, Eng E, Kim E, Peruvian E, Copel T, Ballermann B. Expression of SET, an inhibitor of protein phosphatase 2A, in renal development and Wilms' tumor. *Journal of the American Society of Nephrology* 1998;9(10):1873-1880.
270. Li M, Makkinje A, Damuni Z. The Myeloid Leukemia-associated Protein SET Is a Potent Inhibitor of Protein Phosphatase 2A. *Journal of Biological Chemistry.* 1996;271(19):11059-11062.
271. Junttila MR, Puustinen P, Niemela M, et al. CIP2A inhibits PP2A in human malignancies. *Cell.* 2007;130(1):51-62.
272. Li W, Ge Z, Liu C, et al. CIP2A is overexpressed in gastric cancer and its depletion leads to impaired clonogenicity, senescence, or differentiation of tumor cells. *Clinical Cancer Research.* 2008;14(12):3722-3728.
273. Kerosuo L, Fox H, Perälä N, et al. CIP2A increases self-renewal and is linked to Myc in neural progenitor cells. *Differentiation.* 2010;80(1):68-77.
274. Brault L, Gasser C, Bracher F, Huber K, Knapp S, Schwaller J. PIM serine/threonine kinases in the pathogenesis and therapy of hematologic malignancies and solid cancers. *Haematologica.* 2010;95(6):1004-1015.
275. Chen KF, Liu CY, Lin YC, et al. CIP2A mediates effects of bortezomib on phospho-Akt and apoptosis in hepatocellular carcinoma cells. *Oncogene.* 2010;29(47):6257-6266.
276. Wang J, Anderson PD, Luo W, Gius D, Roh M, SA. A. Pim1 kinase is required to maintain tumorigenicity in MYC-expressing prostate cancer cells. *Oncogene.* 2011;Epub ahead of print.
277. Vaarala MH, Väisänen MR, A. R. CIP2A expression is increased in prostate cancer. *Journal of experimental and Clinical Cancer Reasearch.* 2010;29(1):136.
278. Nieborowska-Skorska M, Hoser G, Kossev P, Wasik MA, Skorski T. Complementary functions of the antiapoptotic protein A1 and serine/threonine kinase pim-1 in the BCR/ABL-mediated leukaemogenesis. *Blood.* 2002;99:4531-4539.
279. Broughton CM, Sherrington P, Pender NT, Clark RE. Molecular status of individual CFU-GM colonies derived from chemotherapy-mobilised peripheral blood stem cells in chronic myeloid leukaemia *Genes, Chromosomes and Cancer.* 1997;18(4):292-298.
280. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2-[Delta][Delta]CT Method. *Methods.* 2001;25(4):402-408.
281. Kubonishi I, I M. Establishment of a Ph1 chromosome-positive cell line from chronic myelogenous leukemia in blast crisis. *International Journal of Cell Cloning.* 1983;1:105-117.
282. Lozzio BB, CB. L. Properties of the K562 cell line derived from a patient with chronic myeloid leukemia. *International Journal of Cancer* 1977;19(1):136.
283. Sundstrom C, Nilsson K. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). *International Journal of Cancer.* 1976;17:565-577.
284. Pettitt AR, Sherrington PD, Cawley JC. The effect of p53 dysfunction on purine analogue cytotoxicity in chronic lymphocytic leukaemia. *British Journal of Haematology.* 1999;106(4):1049-1051.
285. Laemmli. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 1970;227(5259):680-685.
286. Scheinfeld N. Imatinib mesylate and dermatology part 2: a review of the cutaneous side effects of imatinib mesylate. *Journal of Drugs in Dermatology.* 2006;5(3):228-231.
287. de Lavallade H, Apperley JF, Khorashad JS, et al. Imatinib for Newly Diagnosed Patients With Chronic Myeloid Leukemia: Incidence of Sustained Responses in an Intention-to-Treat Analysis. *Journal of Clinical Oncology.* 2008;26(20):3358-3363.
288. Kantarjian HM, Talpaz M, O'Brien S, et al. Survival benefit with imatinib mesylate versus interferon-{alpha}-based regimens in newly diagnosed chronic-phase chronic myelogenous leukemia. *Blood.* 2006;108(6):1835-1840.

289. O'Brien SG, Guilhot F, Goldman JM, et al. International Randomized Study of Interferon Versus STI571 (IRIS) 7-Year Follow-up: Sustained Survival, Low Rate of Transformation and Increased Rate of Major Molecular Response (MMR) in Patients (pts) with Newly Diagnosed Chronic Myeloid Leukemia in Chronic Phase (CMLCP) Treated with Imatinib (IM). *ASH Annual Meeting Abstracts*. 2008;112(11):186-.
290. Gallipoli P, Shepherd P, Drummond M, Holyoake T. Retrospective analysis of prognostic factors and outcome in an imatinib treated CML population from west of Scotland and Lothian. *Haematologica*. 2010;95(Supplement 1):Abstract 818.
291. Branford S, Rudzki Z, Walsh S, et al. High frequency of point mutations clustered within the adenosine triphosphate-binding region of BCR/ABL in patients with chronic myeloid leukemia or Ph-positive acute lymphoblastic leukemia who develop imatinib (STI571) resistance. *Blood*. 2002;99(9):3472-3475.
292. Weisberg E, Griffin JD. Mechanism of resistance to the ABL tyrosine kinase inhibitor STI571 in BCR/ABL-transformed hematopoietic cell lines. *Blood*. 2000;95(11):3498-3505.
293. White DL, Saunders VA, Dang P, et al. Most CML patients who have a suboptimal response to imatinib have low OCT-1 activity: higher doses of imatinib may overcome the negative impact of low OCT-1 activity. *Blood*. 2007;110(12):4064-4072.
294. Hamilton A, Alhashimi F, Myssina S, Jorgensen HG, Holyoake TL. Optimization of methods for the detection of BCR-ABL activity in Philadelphia-positive cells. *Experimental Hematology*. 2009;37(3):395-401.
295. Zola H, Neoh SH, Mantzioris BX, Webster J, Loughnan MS. Detection by immunofluorescence of surface molecules present in low copy numbers. High sensitivity staining and calibration of flow cytometer. *Journal of Immunology Methods*. 1990;135(1-2):247-255.
296. Nichols GL, Raines MA, Vera JC, Lacomis L, Tempst P, DW. G. Identification of CRKL as the constitutively phosphorylated 39-kD tyrosine phosphoprotein in chronic myelogenous leukemia cells. *Blood*. 1994;84(9):2912-2918.
297. Widmer N, Rumpold H, Untergasser G, Fayet A, Buclin T, Decosterd LA. Resistance reversal by RNAi silencing of MDR1 in CML cells associated with increase in imatinib intracellular levels. *Leukaemia*. 2007;21:1561-1562.
298. Davies A, Jordanides NE, Giannoudis A, et al. Nilotinib concentration in cell lines and primary CD34+ chronic myeloid leukemia cells is not mediated by active uptake or efflux by major drug transporters. *Leukaemia*. 2009;23:1999-2006
299. O'Hare T, Walters DK, Stoffregen EP, et al. In vitro Activity of Bcr-Abl Inhibitors AMN107 and BMS-354825 against Clinically Relevant Imatinib-Resistant Abl Kinase Domain Mutants. *Cancer Research*. 2005;65(11):4500-4505.
300. Lucas CM, Fagan JL, Carter A, et al. Rapid diagnosis of chronic myeloid leukemia by flow cytometric detection of BCR-ABL1 protein. *Haematologica*. 2011;96(7):1077-1078.
301. Hiwase DK, Saunders V, Hewett D, et al. Dasatinib Cellular Uptake and Efflux in Chronic Myeloid Leukemia Cells: Therapeutic Implications. *Clinical Cancer Research*. 2008;14(12):3881-3888.
302. Lucas CM, Harris RJ, Giannoudis A, et al. Chronic myeloid leukaemia patients with the e13a2 BCR-ABL fusion transcript have inferior responses to imatinib than e14a2 patients. *Haematologica*. 2009;94(10):1362-1367
303. Lucas CM, Harris RJ, Giannoudis A, Knight K, Watmough SJ, Clark RE. BCR-ABL1 tyrosine kinase activity at diagnosis, as determined via the pCrkL/CrkL ratio, is predictive of clinical outcome in chronic myeloid leukaemia. *British Journal of Haematology*. 2010;149(3):458-460.
304. Allen JC, Talab F, Zuzel M, Lin K, Slupsky JR. c-Abl regulates Mcl-1 gene expression in chronic lymphocytic leukemia cells. *Blood*. 2011;117(8):2414-2422.

305. Li X, Yang J, Chen X, et al. A report of early cytogenetic response to imatinib in two patients with chronic myeloid leukemia at accelerated phase and carrying the e19a2 BCR-ABL transcript. *Cancer Genetics and Cytogenetics*. 2007;176(2):166-168.
306. Andrikovics H, Nahajevszky S, Szilvási A, et al. First and second line imatinib treatment in chronic myelogenous leukemia patients expressing rare e1a2 or e19a2 BCR–ABL transcripts. *Hematological Oncology*. 2007;25(3):143-147.
307. Mondal BC, Majumdar S, Dasgupta UB, Chaudhuri U, Chakrabarti P, Bhattacharyya S. e19a2 BCR-ABL fusion transcript in typical chronic myeloid leukaemia: a report of two cases. *Journal of Clinical Pathology*. 2006;59(10):1102-1103.
308. Hochhaus A, Reiter A, Skladny H, Melo JV, Sick C, Berger U. A novel BCR-ABL fusion gene (e6a2) in a patient with Philadelphia chromosome-negative chronic myelogenous leukemia. *Blood*. 1996;88:2236-2240.
309. Otazu I, Belen R, Olicio R, Pinto A, Zalcberg I, Seuanez H. A rare, in-frame BCR-ABL fusion (e13a3) in a patient with an aggressive chronic myeloid leukaemia. *Acta Haematol*. 2002;108(3):150-153.
310. Snyder DS, McMahon R, Cohen SR, ML. S. Chronic myeloid leukemia with an e13a3 BCR-ABL fusion: benign course responsive to imatinib with an RT-PCR advisory. *American Journal of Hematology*. 2004;75(2):92-95.
311. Meissner R, Covas D, Dias P, Job F, Leite M, Nardi N. Analysis of mRNA transcripts in chronic myeloid leukemia patients. *Genetics and Molecular Biology*. 1999;22(4):475-479.
312. Adler R, Viehmann S, Kuhlisch E, et al. Correlation of BCR/ABL transcript variants with patients' characteristics in childhood chronic myeloid leukaemia. *European Journal of Haematology*. 2008;82(2):112-118.
313. Prejzner W. Relationship of the BCR gene breakpoint and the type of BCR/ABL transcript to clinical course, prognostic indexes and survival in patients with chronic myeloid leukemia. *Medicine Science Monitor*. 2002;8(5):193-197.
314. Jaubert J, Martiat P, Dowding C, Ifrah N, Goldman J. The position of the M-BCR breakpoint does not predict the duration of chronic phase or survival in chronic myeloid leukaemia. *British Journal of Haematology*. 1990;74(1):30-35.
315. Tefferi A, Bren G, Wagner K, Schaid D, Ash R, Thibodeau S. The location of the Philadelphia chromosomal breakpoint site and prognosis in chronic granulocytic leukemia. *Leukemia*. 1990;4(12):839-842.
316. Suttorp M, Thiede C, Tauer JT, Range U, Schlegelberger B, von Neuhoff N. Impact of the type of the BCR-ABL fusion transcript on the molecular response in pediatric patients with chronic myeloid leukemia. *Haematologica*. 2010;95(5):852-853.
317. Castagnetti F, Gugliotta G, Palandri F, et al. BCR-ABL fusion transcript and outcome of chronic myeloid leukemia patients in early chronic phase treated with imatinib: a GIMEMA CML WP analysis. *Haematologica*. 2011;96(Supplement 2):487A.
318. Lyons MA, Wittenburg H. Susceptibility to cholesterol gallstone formation: Evidence that LITH genes also encode immune-related factors. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*. 2006;1761(10):1133-1147.
319. Chen J, Martin BL, Brautigan DL. Regulation of protein serine-threonine phosphatase type-2A by tyrosine phosphorylation. *Journal of Biological Chemistry*. 1992;257(5074):1261-1264.
320. Blick M, Romero P, Talpaz M, et al. Molecular characteristics of chronic myelogenous leukemia in blast crisis. *Cancer Genetics and Cytogenetics*. 1987;27(2):349-356.
321. McCarthy DM, Rassool FV, Goldman JM, Graham SV, GD. B. Genomic alterations involving the c-myc proto-oncogene locus during the evolution of a case of chronic granulocytic leukaemia. *Lancet*. 1984;15(2):1362-1365.

322. Guilhot F, Mahon FX, Guilhot J, et al. Randomized comparison of imatinib versus imatinib combination therapies in newly diagnosed chronic myeloid leukaemia (CML) patients in chronic phase (CP): First results of the phase III (SPIRIT) trial from the French CML Group (FI LMC) *Blood* 2008;112(74): abstract 183.
323. Kickstein E, Krauss S, Thornhill P, et al. Biguanide metformin acts on tau phosphorylation via mTOR/protein phosphatase 2A (PP2A) signaling. *PNAS.* 2010;107(50):21830-21835.
324. Szczepanski T, Orfao A, van der Velden VHJ, San Miguel JF, van Dongen JJM. Minimal residual disease in leukaemia patients. *Lancet Oncology.* 2001;2:409-417.
325. Hughes TP, Hochhaus A, Saglio G, et al. ENESTnd Update: Continued Superiority of Nilotinib Versus Imatinib In Patients with Newly Diagnosed Chronic Myeloid Leukemia In Chronic Phase (CML-CP) *Blood.* 2010;116(21):207A.
326. Calabretta B, Perrotti D. The biology of CML blast crisis. *Blood.* 2004;103(11):4010-4022.
327. Ma J, Arnold HK, Lilly M, Sears R, Kraft A. Negative regulation of Pim-1 protein kinase levels by the B56[beta] subunit of PP2A. *Oncogene.* 2007;26(35):5145-5153.
328. Gallipoli P, Shepherd P, Irvine D, Drummond M, Holyoake T. Restricted access to second generation tyrosine kinase inhibitors in the UK could result in suboptimal treatment for almost half of chronic myeloid leukaemia patients: results from a West of Scotland and Lothian population study. *British Journal of Haematology.* 2011.
329. Bockelman C, Hagstrom J, Makinen LK, et al. High CIP2A immunoreactivity is an independent prognostic indicator in early-stage tongue cancer. *British Journal of Cancer.* 2011;104(12):1890-1895.
330. Dong Q-Z, Wang Y, Dong X-J, et al. CIP2A is Overexpressed in Non-Small Cell Lung Cancer and Correlates with poor prognosis. *Annals of Surgical Oncology* 2010:1-9.
331. Ma L, Wen Z, Liu Z, et al. Overexpression and Small Molecule-Triggered Downregulation of CIP2A in Lung Cancer. *PLoS ONE.* 2011;6(5):e20159.
332. Katz J, Jakymiw A, Ducksworth MK, et al. CIP2A expression and localization in oral carcinoma and dysplasia. *Cancer therapy and biology.* 2010;10(7):694 - 699.
333. Qu W, Li W, Wei L, Xing L, Wang X, Yu J. CIP2A is overexpressed in esophageal squamous cell carcinoma. *Medical Oncology.* 2010:1-6.
334. Liu J, Wang X, Zhou G, et al. Cancerous inhibitor of protein phosphatase 2A is overexpressed in cervical cancer and upregulated by human papillomavirus 16 E7 oncoprotein. *Gynecologic Oncology.* 2011;In Press, Corrected Proof.
335. Wang J, Li W, Li L, Yu X, Jia J, Chen C. CIP2A is over-expressed in acute myeloid leukaemia and associated with HL60 cells proliferation and differentiation. *International Journal of Laboratory Hematology.* 2011;33(3):290-298
336. Lê LH, Erlichman C, Pillon L, et al. Phase I and Pharmacokinetic Study of Fostriecin given as an Intravenous Bolus Daily for Five Consecutive Days. *Investigational New Drugs.* 2004;22(2):159-167.
337. Neviani P, Santhanam R, Oaks J, et al. FTY720, a new alternative for treating blast crisis chronic myelogenous leukemia and Philadelphia chromosome-positive acute lymphocytic leukemia. *Journal of Clinical Investigation.* 2007;117(9):2408–2421.
338. Felsher DW, Bishop JM. Reversible Tumorigenesis by MYC in Hematopoietic Lineages. *Molecular Cell.* 1999;4(2):199-207.
339. Wang H, Mannava S, Grachtchouk V, et al. c-Myc depletion inhibits proliferation of human tumor cells at various stages of the cell cycle. *Oncogene.* 2007;27(13):1905-1915.
340. Davis AC, Wims M, Spotts GD, Hann SR, Bradley A. A null c-myc mutation causes lethality before 10.5 days of gestation in homozygotes and reduced fertility in heterozygous female mice. *Genes & Development.* 1993;7(4):671-682.

341. Baudino TA, McKay C, Pendeville-Samain H, et al. c-Myc is essential for vasculogenesis and angiogenesis during development and tumor progression. *Genes & Development*. 2002;16(19):2530-2543.
342. Bazeos A, Marin D, Reid AG, et al. hOCT1 transcript levels and single nucleotide polymorphisms as predictive factors for response to imatinib in chronic myeloid leukemia. *Leukemia*. 2010;24(6):1243-1245.
343. White DL, Dang P, Engler J, et al. Functional Activity of the OCT-1 Protein Is Predictive of Long-Term Outcome in Patients With Chronic-Phase Chronic Myeloid Leukemia Treated With Imatinib. *Journal of Clinical Oncology*. 2010;28(16):2761-2767.
344. Argetsinger LS, Kouadio JL, Steen H, Stensballe A, Jensen ON, Carter-Su C. Autophosphorylation of JAK2 on tyrosines 221 and 570 regulates its activity. *Molecular Cell Biology*. 2004;24:4955-4967.
345. Huang HM, Lin YL, Chen CH, Chang TW. Simultaneous activation of JAK1 and JAK2 confers IL-3 independent growth on Ba/F3 pro-B cells. *Journal of Cell Biochemistry*. 2005;96:361-375.
346. Xie S, Lin H, Sun T, Arlinghaus RB. Jak2 is involved in c-Myc induction by Bcr-Abl. *Oncogene*. 2002;21:7137-7146.
347. Lucas CM, Harris RJ, Giannoudis A, Davies A, Clark RE. SET Binding Protein 1 expression does not predict clinical outcome in chronic myeloid leukaemia. *eBlood*. 2010.
348. Horita M, Andreu EJ, Benito A, et al. Blockade of the Bcr-Abl Kinase Activity Induces Apoptosis of Chronic Myelogenous Leukemia Cells by Suppressing Signal Transducer and Activator of Transcription 5-dependent Expression of Bcl-xL. *The Journal of Experimental Medicine*. 2000;191(6):977-984.
349. Klejman A, Schreiner SJ, Nieborowska-Skorska M, Slupianek A, Wilson M, Smithgall TE. The Src family kinase Hck couples BCR/ABL to STAT5 activation in myeloid leukemia cells. *EMBO J*. 2002;21:5766-5774.